PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(54) Title: IDENTIFICATION OF GENES HAVING A ROLE IN THE PRESENTATION OF DIABETIC NEPHROPATHY

(57) Abstract

A method for identifying a gene having a role in the presentation of diabetic nephropathy comprises culturing mesangial cells in the presence of a concentration of glucose sufficient to induce differential expression, especially up–regulation, of a gene susceptible to such differential expression and identifying the gene so induced. The cells are also optionally subjected to mechanical strain and/or TGF–β1 can be added to the culture medium. The differentially expressed genes can be identified by suppression subtractive hybridisation. The method has resulted in the identification of novel genes which play a role in the presentation of diabetic nephropathy. The genes can be used as diagnostic markers for diabetic nephropathy and as the basis of drug development programmes.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav	TM	Turkmenistan
BF	Burkina Faso	GR	Greece		Republic of Macedonia	TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's	NZ	New Zealand		
CM	Cameroon		Republic of Korea	PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

Description

Identification of genes having a role in the presentation of diabetic nephropathy.

Technical Field

This invention relates to the characterisation and identification of genes which play a role in diabetes, more particularly in the onset and progression of diabetic nephropathy and to the use of genes so characterised and/or identified as diagnostic markers for diabetic nephropathy and as the basis of drug development programmes.

Background Art

Between 2-5% of the population develops diabetes mellitus and 20-30% of diabetics develop diabetic nephropathy.The latter accounts for over 30 % of end-stage renal failure (E.S.R.F.) requiring dialysis or transplantation in western society. The pathological hallmark of diabetic nephropathy is glomerulosclerosis due to accumulation of extracellular matrix proteins in the glomerular mesangium. Mesangial matrix accumulation reflects both increased synthesis and decreased degradation of extracellular matrix (ECM) components, and correlates with the clinical onset of proteinuria, hypertension and progressive kidney failure. Hyperglycaemia is a major stimulus for mesangial cell matrix production in diabetic nephropathy. The mechanisms by which hyperglycaemia perturb mesangial cell function are still being

appreciated and include direct effects of high extracellular glucose levels and indirect effects transduced through alterations in glomerular haemodynamics and through the actions of advanced glycosylation end products.

Propagation of mesangial cells under conditions of high ambient glucose has proved a useful *in vitro* model with which to probe the molecular basis for mesangial matrix accumulation in diabetes, attributable to hyperglycaemia. Specifically, exposure of cultured mesangial cells to high glucose stimulates *de novo* synthesis of ECM components, such as type IV collagen, fibronectin and laminin, and other products that are accumulated *in vivo* (Ayo, S.H., *et al.* (1990) *Am.J. Pathol. 136*, 1339-1348; Wahab, N.A., *et al.* (1996) *Biochem. J. 316*, 985-992; and Ayo, S.H., *et al.* (1991) *Am. J. Physiol. 260*, F185-F191).

In view of the high morbidity and mortality rate from diabetic nephropathy in diabetics there is a need to identify stimuli which affect the onset and progression of diabetic nephropathy with the aim of preventing such onset or inhibiting or limiting the progression thereof.

Disclosure of Invention

The invention provides a method for identifying a gene having a role in the presentation of diabetic nephropathy, which method comprises culturing mesangial cells in a medium in the presence of a concentration of glucose sufficient to induce differential expression of a

gene susceptible to such differential expression and identifying the gene
so induced.

Preferably, the mesangial cells are cultured in the presence of a
concentration of glucose sufficient to induce up-regulation of a gene
susceptible to such up-regulation.

Further, preferably, the concentration of glucose is greater than 5
mM.

A concentration of 5 mM falls within the normal range of
plasma glucose levels in a healthy human subject (4.2 – 6.4 mmol/l).

The concentration of glucose used is suitably in the range 5-30
mM. The concentration of 30 mM was chosen as the classic *"in vitro"*
model of diabetic nephropathy which induces changes in mesangial
function that mimic human disease. This level is also encountered in
many diabetics *in vivo.*

In one embodiment, the mesangial cells are subjected to
mechanical strain.

In a further embodiment, transforming growth factor β1 (TGF-
β1) is added to the culture medium.

Preferably, the differentially expressed gene is identified by
suppression subtractive hybridisation.

Suppression subtractive hybridisation (SSH) is a method based on suppressive PCR that allows creation of subtracted cDNA libraries for the identification of genes differentially expressed in response to an experimental stimulus (Gurskaya, N.G., *et al.* (1996) *Anal. Biochem. 240*, 90-97). SSH differs from earlier subtractive methods by including a normalisation step that equalises for relative abundance of cDNAs within a target population. This modification should enhance the probability of identifying increased expression of low abundance transcripts, and represents a potential advantage over other methods for identifying differentially regulated genes such as differential display-PCR (DD-PCR) (Liang, P., and Pardee, A.B. (1992) *Science 257*, 967-97) and cDNA-representation difference analysis (Hubank, M., and Schatz, D.G., (1994) *Nucleic Acid Res. 22*, 5640-5648).

To date we have used SSH to identify 150 genes differentially induced when human mesangial cells were exposed to high extracellular glucose (defined herein as 30 mM versus 5 mM) *in vitro*. These genes included:

(a) known regulators of mesangial cell activation in diabetic nephropathy, namely fibronectin, caldesmon, thrombospondin and plasminogen activator inhibitor-1;

(b) novel genes; and

5

(c) genes whose induction by high glucose has not previously been reported as hereinafter described.

Prominent among the latter were genes encoding cytoskeleton-associated proteins and connective tissue growth factor (CTGF), a modulator of fibroblast matrix production. We have also demonstrated elevated CTGF mRNA levels in glomeruli of rats with streptozotocin-induced diabetic nephropathy.

In one aspect of the invention, the possibility of differential expression due to hyperosmolarity is excluded.

Hyperosmolarity is, however, a component of diabetic nephropathy and thus hyperosmolarity may represent a mechanism through which high glucose induces differential expression of certain genes having a role in the presentation of the disease.

For example, we have shown that mannitol provoked less mesangial cell CTGF expression *in vitro* than high glucose, excluding hyperosmolarity as the key stimulus.

High glucose also stimulated expression of transforming growth factor $\beta 1$ (TGF-$\beta 1$) and addition of TGF-$\beta 1$ to mesangial cells triggered CTGF expression. Anti-TGF-$\beta 1$ antibody blunted CTGF expression induced by high glucose. Together, these data suggest that (1) high glucose stimulates mesangial CTGF expression by TGF$\beta 1$-dependent

6

and independent pathways, and (2) CTGF may be a mediator of TGF-
β1-driven matrix production within a diabetic milieu.

CTGF may therefore be an attractive target for design of novel
anti-sclerotic therapies for diabetic glomerulosclerosis.

CTGF derived from mesangial cells is a potential stimulus for
increased synthesis of ECM proteins and mesangial expansion in
diabetic nephropathy. The mechanisms by which high glucose triggers
mesangial cell CTGF and, indeed, TGF-β, mRNA expression remain to
be defined. Possible upstream triggers of CTGF transcription in
response to high glucose include *de novo* synthesis of diacylglcerol
(DAG) and subsequent activation of protein kinase C (PKC)
(DeRubertis, F.R., and Craven, P., (1994) *Diabetes 43*, 1-8 and Fumo P.,
et al. (1994) *Am. J. Physiol. 267*, F632-F638), non-enzymatic glycation
end-products (Brownlee, M., *et al.* (1984) *Ann. Intern. Med 101*, 527-
537 and Cohen, M.P., and Ziyadeh, F.N. (1994) *Kidney Int. 45*, 475-
484) increased activity of the polyol pathway and disordered
myoinositol metabolism (Goldfarb, S., *et al.* (1991) *Diabetes 40*, 465-
471 1991) or through the recruitment of locally generated growth
factors such as TGF-β1 and other mediators. (Sharma K., and Ziyadeh,
F.N., (1995) *Diabetes 44*, 1139-1146).

TGF-β1 has been implicated as the key mediator of extracellular
matrix accumulation in diabetic nephropathy and other chronic renal
disease . Several studies have reported increased expression of TGF-β1
in renal glomeruli in human and experimental models of diabetes (Park,

I., *et al.* (1997) *Diabetes 46*, 473-480; Sharma, K., (1996) *Diabetes 45*, 522-530). Short term administration of TGF-β1 neutralising antibodies attenuates overexpression of mRNAs encoding matrix components and glomerulosclerosis in the STZ mouse model of diabetes (Park, I., *et al.* (1997) *Diabetes 46*, 473-480). CTGF shares some of the biological actions of TGF-β1 such as stimulation of cell proliferation and extracellular matrix protein synthesis in fibroblasts. When considered in this context, the results described herein suggest that TGF-β1 may promote mesangial matrix production, in part, by inducing CTGF synthesis. TGF-β1 has a complex profile of biological activities that includes pro-inflammatory, pro-fibrotic and anti-inflammatory effects. By targeting CTGF it may be possible to attenuate the sclerosis-inducing effects of TGF-β1 while preserving its more desirable anti-inflammatory activities.

Novel genes identified by the method according to the invention are identified herein as IHG (*I*ncreased in *H*igh *G*lucose) and DHG (*D*own in *H*igh *G*lucose) and are represented by genes which include the following sequences 1, 3 and 4:

1)TTGGAATAGTTCTTGCTTTATAAAAATAGTACTGCGATTAAA
AAAAAAGCACTTCTGCCAAAGGAACCATGTTCCAACACCGCA
AACAAGGTGTTCTGCTTAAACAGAGTAAGATACACCACCCCC
ATCCATCCTTCCTTCCCTGTTCCCCTCCCAACTTGAGTTGTGT
CATTCGCACCAGTGTCCTGGGTGGTAGGGATGCTACAGCCAC
CTAAGGCAAGGAGCCCTGGGAGGTGGGAGGGCTTGCATGGTT
AAGCACACCAGAACTGAAGCGCAAAAGGGTCAGCTGTCTTCA

TCTAGAATCTCTGGATGTTCCTTCCAGAAAGCATCCCCGATGA

TATCGCAGTGCAAGGGCACTGGCTTTGTCCTGGTCCGGGTCAC

TGCCATCTTTTTCCTTCCATTTCTGTTGGCAGCTTAATTTCTTT

TGTCATCACTTCATCCACCTTCTGCCATATCAACACAGTCCCTT

5   TCCTATACATCGGCAGCTCATTATTATAGTTGATGTTGAATTC

AGAAACAAAATCTCATTCTTGTCTGCTGNAAGAGTTCCCTGT

AATCTCCCTTGGGCTTGTACTGGTGTTAGTCCAGATTGTTG

(SEQ ID NO: 1)

....

10   ....GGTCCTTTAA

AGTCTGGTTGCTGGGATACACCACGACTCTTCCGGTCAAAGCC

TGGGGGATACAGAAGGGGCTRGTCCTCAAAGTAATCCCGCCA

ATAAAACAYATAGCTGGAGGCAAACTGGGAGGYCACGTGAGT

CATGAACTTTACTGGCTCTTCTTTTAAACCAATTGGTTTTCCGC

15   TTGWACACAAAGCTGTACTCATCACTCTGTCCATAACGCGAT

CACAATATCCTCTAGTTCTTCCATCACAGTCTGCGCACATTTG

GTCATCAGCTGGAGAGCACGGCTGTCATTGGGTTTTGCAAAGT

TGTGCTTCTCAGCAAACCGATGGAAATTCCGGCCGTCCAGCCG

NACTACCACCCAGCAGTGTGCCAGGCAGGTGTCGTCAGCCTC

20   GAAGTCCCTCACGTACTCGAACTTGCTTTTGCCATGGTCGCC

CCCAATCTCAGGTACCGTCTCAGAGTGATGGAAATGGTGGCC

AAGGAATCGTGAACCTTAACTTTACAGGCGCCCCACATTCTAC

ACGCGGAAAGGAAAGGGCCAGATAGCCCCGCCCCGGAAGTG

TTCTCTTCGTGGCTACTCTAGCCGTAGGGCGGTCATAGTCTCT

25   CTCGSCTCTCCCTGKAGTTCTTAAMCYYCCAGGGAAARAGGA

TGGAGGTTTAGGTTCCTCCGTTAGCACCTTCCACGCTTGCTTCT

TCCTCCTCCCGGTCTGCGGCAAATCAGTCTCACGAGGTTTTTA

AAAATTATTTTTATCTGCTGGCCTT (SEQ ID NO: 2)

....ATGACACAAATATTAG

GATTTTATTTTTACTATTATCCACCAGCAACAAGATATCAAAC

ACTGGTTCTGTGATTATTTAATGGTGAAAAAGTTGAATAAATC

AATTTAGTATACCCATATGTTGGAATATTGAGTCCATTTTTCTT

TTAAAAATCACACTTTGGAATAATTGATGATACTGGCAAATGC

TCAAGCTGAGTGGAAAAATATATAAACATTGTATAGGCGAAT

AATTCCAATCTTGTGCATTCCCTGTGTAAACCTACATACACAA

AAAGAAAAAGACTGAAAGGAACCATCCACAATGCTTTGATC

GGGAAAGACGGAGAAACAAAGTGTTAATTTTCTTAACTATAG

TTTTNGGTGTATTCCAGATTTTCTACAAGTTAATA (IHG-1) (SEQ

ID NO: 3);

GTACTTTGGATTTGGTTAACCTGTTTTCTTCAAGCCTGAGGTTT

TATATACAAACTCCCTGAATACTCTTTTTGCCTTGTATCTTCTC

AGCCTCCTAGCCAAGTCCTATGTAATATGGAAAACAAACACT

GCAGACTTGAGATTCAGTTGCCGATCAAGGCTCTGGCATTCAG

AGAACCCTTGCAACTCGAGAAGCTGTTTTTATTTCGTTTTTGTT

TTGATCCAGTGCTCTCCCATCTAACAACTAAACAGGAGCCATT

TCAAGGCGGGAGATATTTAAACACCCAAAATGGTTGGGTCT

GATTTTCAAACTTTTAAAACTTCACTACTGATGATTCTGCACG

CTAAGGCGAATTTGGTCCAAACACATAAGTGTGTGTGTTTTGT

ATACACTGTATGACCCCACCCCAAATCTTTGTATTGTCCACAT

TCTCC (SEQ ID NO: 4);

25

3)AGAAGCAATTTAGGAANCCNACAGNAAANAAATGCTGTTTT

ATAGGAGAGAAAACACGGCACACCAAGGTTAAGTAGTTTGTA

10

GACGATGTTGAATAGGTTCAGGTACAGGTCAATGCAGTGATG
AGGAAAGCACCTANGTATACTTGACAGATAGTCCCCTTTGCTT
AACACCCAACTCCTCCACCCTGTGCAGTTTNNCTTGTGCCAGT
GATCACAGGATTCGCTGAGTGAATTACCATAATTGGATTTAAT
5   TCACGAAGGGGATGTTTTC (IHG-3) (SEQ ID NO: 5); and

4)ATTGATAGAGGCCCTGTTTCATGACATTTCATGAGTTTCAAT
ATGTTGTTCAGCATGTTGTGAGGTGACTCTCAGCCCCTTTCCC
ACTGAGATGGACTGTGGTGATGCTGTGAGGGTGTGACTGACA
10   CACCTTCATGTGCCCAAGCATGGGTTTGATCACAGGTCACATG
CAGTTTTTGGCATAGTAAATGTATCATTGTTCTTTTCCTCCCTC
CTAAAGGAAACAGAGGAATCCACCTGTATGAGAGTGCCATGT
AGGGATAAACTTAAAGGACAGATGACACATTGGTCATGTTCG
TGATAAGGAAA (DHG-1) (SEQ ID NO: 6).
15

The invention also provides genes containing sequences 1-3, 5 and 6 set out above.

In initial studies the gene IHG-2 was assumed to be new.
20   However, as hereinafter demonstrated IHG-2 was identified as being a formerly unknown part of the gremlin gene. We have found that mesangial cell gremlin mRNA levels are induced by high glucose, cyclic mechanical strain and TGF-β1 *in vitro,* and gremlin mRNA levels are elevated in the renal cortex of rats with streptozotocin-induced
25   diabetic nephropathy *in vivo.* Gremlin expression was observed in parallel with induction of bone morphogenetic protein-2 (BMP-2), a target for gremlin in models of cell differentiation.

Gremlin, together with DAN and cerberus, are members of the cysteine knot super-family of proteins that have recently been shown to play important roles in limb development and neural crest cell
5   differentiation (Hsu, D.R., *et al.* (1998) *Mol. Cell.* 1, 673-83; Zuniga, A., *et al.* (1999) *Nature* 401, 598-602). Of potential interest in the context of diabetic nephropathy, gremlin is a putative inhibitor of BMP-2 in models of neural crest cell differentiation. BMP-2 has recently been reported to have antiproliferative effects on mesangial
10   cells.

The following Examples show that (a) IHG-2 is part of gremlin, (b) gremlin is expressed in diabetic nephropathy *in vivo*, (c) both glycemic and mechanical strain stimulate mesangial cell gremlin
15   expression *in vitro*, (d) high glucose induces gremlin, in part, through TGFβ-mediated pathways, and (e) gremlin is a potential endogenous antagonist of BMPs within a diabetic glomerular milieu.

The invention also provides use of a gene identified by the
20   method according to the invention:

1)   as a diagnostic marker for the progression and presentation of diabetic nephropathy;

25   2)   as an index of disease activity and the rate of progression of diabetic nephropathy; and

3)   as a basis for identifying drugs for use in the prevention and/or therapy of diabetic nephropathy.

Thus, it will be appreciated that early diagnosis of diabetic nephropathy based on diagnostic markers identified in accordance with the invention can be used in conjunction with aggressive therapies to prevent full blown development of diabetic nephropathy.

The level of expression of genes identified in accordance with the invention could correlate with the degree of disease progression.

Furthermore, genes identified in accordance with the invention can represent novel therapeutic targets for drug development programmes. Once it has been established that a given gene has a designated role in the pathophysiology of diabetic nephropathy, the development of new therapeutic agents (such as, for example, small molecules, recombinant inhibitors and receptor antagonists) could be designed to inhibit expression of these genes and, thereby, prevent the development of diabetic nephropathy.

Genes identified in accordance with the invention can also be used as a clinical index of progressive renal sclerosis and scarring, as a guide to the response of progressive diabetic nephropathy to therapy and also as markers of the prevention or development thereof.

It is possible to generate mouse knock-out (k/o) models for genes identified in accordance with the invention and to generate

diabetic k/o mouse models, (for example by treatment with streptozotocin) and determine if onset of diabetic nephropathy is inhibited, reduced or delayed. Thus one can determine if a given knock-out gene has a definite role in the progression and development of diabetic nephropathy.

Brief Description of Drawings

Fig. 1 is an autoradiograph of CTGF levels analysed by Northern Blot as described in Example 2;

Fig. 2 is a graph of the relative amount of CTGF mRNA as estimated by Phosphor Imager quantification as described in Example 2;

Fig. 3 is a 2 % agarose gel showing ethidium-stained PCR products as described in Example 2;

Fig. 4 is a nucleotide sequence alignment of the rat CTGF transcript and the mouse CTGF homologue fisp 12 as described in Example 3;

Fig. 5 is an amino acid sequence alignment of the rat CTGF transcript and the mouse CTGF homologue fisp 12 as described in Example 3;

Fig. 6 is a 2 % agarose gel showing ethidium-stained PCR products as described in Example 3;

Fig. 7 is an autoradiograph of CTGF levels in the presence of
TGF-β1 and TGF-β1 neutralising antibodies analysed by Northern Blot
as described in Example 4;

5

Fig. 8 is a graph of the relative amount of CTGF mRNA as
estimated by Phosphor Imager quantification as described in Example 4;

Fig. 9 is an autoradiograph of CTGF levels in the presence of

10   varying amounts of glucose and TGF-β1 neutralising antibodies
analysed by Northern Blot as described in Example 4;

Fig. 10 is a graph of the relative amount of CTGF mRNA as
estimated by Phosphor Imager quantification as described in Example 4;

15

Fig. 11 is an autoradiograph of CTGF levels in the presence of
varying amounts of glucose and PKC inhibitor GF102903X analysed by
Northern Blot as described in Example 4;

20   Fig. 12 is a graph of the relative amount of CTGF mRNA as
estimated by Phosphor Imager quantification as described in
Example 4;.

Fig. 13 is a graphical representation of a BLAST output from the

25   EST database as described in Example 6;

Fig. 14 is a graphical representation of the alignment of human gremlin and rat drm when compared using the BLAST algorithm as described in Example 6;

5   Fig. 15 is the sequence of mesangial cell gremlin cDNA;

Fig. 16 is an autoradiograph of IHG-2, gremlin, fibronectin and GAPDH mRNA levels analysed by Northern Blot as described in Example 7;

10

Fig. 17 is a graph of relative mRNA levels as estimated by Phosphor Imager quantification as described in Example 7;

Fig. 18 is an autoradiograph of gremlin, fibronectin and GAPDH

15   mRNA analysed by Northern Blot as described in Example 7;

Fig. 19 is a further graph of relative mRNA levels as estimated by Phosphor Imager quantification as described in Example 7;

20   Fig. 20 is an autoradiograph of gremlin mRNA levels in the kidney cortex of a STZ-diabetic rat and an age matched control analysed by Northern Blot as described in Example 7;

Fig. 21 is a further graph of relative mRNA levels as estimated

25   by Phosphor Imager quantification as described in Example 7;

16

Fig. 22 is an autoradiograph of gremlin, fibronectin and GDAPH mRNA levels analysed by Northern Blot as described in Example 8;

Fig. 23 is a graph of relative mRNA levels as estimated by
5   Phosphor Imager quantification; and

Fig. 24 is a graphical representation of representative reactions of four independent experiments as described in Example 9.

10   The invention will be further illustrated by the following Examples:

Modes for Carrying Out the Invention

15   Example 1

Identification of mesangial cell genes differentially induced by high glucose.

20   a) Cell culture and streptozotocin-induced diabetic rats

Primary human mesangial cells were cultured as previously reported (Brady, H.R., *et al.* (1992) *Kidney Int.* *42*, 480-487 and Denton, M.D., *et al.* (1991) *Am. J. Physiol.* *261*, F1071-F1079). Cells (passage
25   7-11) were maintained in medium (Clonetics) containing either 5 mM or 30 mM D-glucose for 7 days. Culture medium was replenished three times during this period to maintain glucose levels in the desired range. To control for the effects of hyperosmolarity, mesangial cells were

cultured in media containing 5 mM glucose supplemented with 25 mM mannitol.

5   Male Munich-Wistar rats (260-290 g, Simonsen Laboratories) were rendered diabetic by treatment with streptozotocin (STZ; Sigma), 50 g/kg, intravenously as described previously (Zatz, R., *et al.* (1985). *Proc Natl. Acad. Sci. USA. 82*, 5963-5967). At months 2 and 4 after induction of diabetic nephropathy (DN), rats were anaesthetized with intraperitoneal injection of pentobarbital (50 mg/kg), and the right 10   kidney was excised and weighed immediately. Glomeruli were isolated from renal cortex by the standard sieving method (Brady, H.R. *et al.* (1992) and Denton, M.D. *et al.* (1991) *supra*). Glomerular isolation was completed within 20 minutes of removing the kidney. RNA extraction proceeded immediately thereafter.

15

b) RNA isolation

Polyadenlyated RNA was isolated from mesangial cells using the Microfast Track (Microfast Track is a Trade Mark) kit (Invitrogen). 20   Total RNA was isolated from glomeruli using RNAzol solution (TEL-test Inc.).

c) Suppression subtractive hybridisation (SSH)

25   SSH was performed with the PCR-SELECT cDNA subtraction kit (Clontech) as directed by the manufacturer with the modification that a four-fold greater than recommended amount of driver cDNA was

added to the second hybridisation. Starting material consisted of 2 µg of mesangial cell mRNA cultured in 30 mM D-glucose for 7 days as "tester" and 2 µg of mesangial cell mRNA cultured in 5 mM D-glucose for 7 days as "driver". Thirty primary PCR cycles and 12 secondary

5   PCR cycles were performed.

d) Cloning and sequencing of cDNAs

PCR products generated by SSH were subcloned into the PCR

10   2.1 vector using the original TA cloning kit (Invitrogen). Subcloned cDNAs were isolated by colony PCR amplification. Sequencing was performed using an automated ABI 370A DNA sequencing system. Sequence reactions were carried out with the ABI prism dye terminator cycle sequencing ready reaction kit (Perkin Elmer). The sequences

15   obtained were compared against GenBank/EMBL and Expressed Sequence Tag (EST) databases using BLAST searches.

SSH analysis suggested differential induction of 16 mRNAs in primary cultures of human mesangial cells propagated for 7 days in 30

20   mM glucose. Northern Blots performed using formaldehyde denaturation according to standard protocols and quantitated using a Phosphor Imager (Biorad) confirmed differential expression of fifteen of the sixteen subcloned fragments as indicated in Table 1.

25   In Table 1 [a] refers to the sequence identity based on comparisons with the Genbank/EMBL database;

b refers to an estimate of the size (kb) of the mRNA identified by
Northern Blot analysis; and

c refers to the differential expression of each gene based on
5   Northern Blot analysis of primary human mesangial cells cultured under
indicated conditions relative to expression in cells cultured in 5 mM
glucose. Values were obtained by Phosphor-Imaging and were
normalised by comparison with glyceraldehyde-3-phosphate
dehydrogenase (GAPDH) (*, detected in mesangial cells cultured in 5
10   mM glucose + 25 mM mannitol and 30 mM glucose, but not in 5 mM
glucose, fold expression is degree of expression relative to that found in
5 mM glucose).

20

Table 1

Summary of cDNAs identified by SSH as being induced in mesangial cells cultured in high glucose.

5

Gene[a]	mRNAkb[b]	Differential Expression[c]	
		30mM glucose	*25mM mannitol+ 5mM glucose*
Extracellular Matrix Proteins			
Fibronectin	7.0	2.1-fold	1.5-fold
Thrombospondin	6.0	7.0-fold	8.0-fold
Actin-Binding Proteins			
MRLC	0.9	3.9-fold	1.6-fold
T-plastin	1.2	4.2-fold	1.8-fold
Caldesmon	3.6	3.3-fold	2.8-fold
Profilin	1.0	2.2-fold	2.3-fold
CAP	2.6	1.5-fold	1.7-fold
ARP3	2.5	2.0-fold	1.0-fold
Growth Factors			
CTGF	2.4	3.0-fold	1.5-fold
Others			
PAI-1	2.0	1.2-fold	1.0-fold
	3.0	3.9-fold	2.0-fold
RBM3	1.5	1.8-fold	1.4-fold
Ubiquitin	3.0	2.3-fold	1.7-fold
TCTP	0.8	4.3-fold	2.5-fold
IHG-1	3.4	*	*
IHG-2	2.5	2.0-fold	2.0-fold

Sequence analysis revealed induction of four genes implicated previously in the pathogenesis of diabetic nephropathy: fibronectin, caldesmon, PAI-1, and thrombospondin. Of eleven other cDNA fragments, one encoded a novel gene, designated herein as IHG-1 and

5   ten encoded known genes, including IHG-2 as hereinafter described, whose induction by high glucose had not been reported previously. Prominent among the latter genes were connective tissue growth factor (CTGF) and several cytoskeleton-associated proteins, namely profilin, caldesmon, adenyl cyclase-associated protein (CAP), actin-related

10   protein-3 (ARP3), T-plastin, and myosin regulatory light chain (MRLC). Subsequent studies focused on induction of CTGF, a regulator of matrix production in several model systems as described in Examples 2 and 3. The prominence of genes encoding multiple actin-binding proteins is also noteworthy, given recent reports implicating F-

15   actin disassembly in the pathogenesis of mesangial cell dysfunction and glomerular hypertension in diabetic nephropathy (Zhou, X., *et al.* (1995) *Lab. Invest. 73*, 372-383 and Zhou, X. Lai, *et al.* (1997) *Kidney Int. 51*, 1797-1808). The induction of profilin expression is particularly interesting given its role as a regulator of actin polymerization under

20   conditions of cell stress (Sohn, R.H. and Goldschmidt-Clermont, P.J. (1994) *Bioessays 16*, 465-472).

Within a diabetic milieu, high glucose levels may perturb cellular function through glucose-specific actions or by increasing the

25   osmolarity of extracellular fluids. The role of hyperosmolarity as a mediator of gene induction by high glucose was assessed by comparing mRNA levels, as determined by Northern Blot, in cells cultured in either

30 mM glucose or in 5 mM glucose supplemented with 25 mM mannitol. High glucose was more effective than high osmolarity at inducing expression of CTGF, myosin regulatory light chain (MRLC), actin related protein 3 (ARP3), T-plastin and translationally controlled

5   tumor protein (TCTP). High glucose and mannitol-induced hyperosmolarity afforded equivalent induction of the other products.

Example 2

10   CTGF expression in mesangial cells cultured in high glucose.

a) Influence of high ambient glucose on CTGF mRNA levels in human mesangial cells.

15   CTGF, is a 38kD cysteine-rich secreted peptide known to modulate ECM production in some extrarenal cell types. In Example 1, SSH analysis identified a cDNA fragment of 250 bp which was identical to bases 814-1061 of the human CTGF cDNA. Induction of CTGF mRNA expression in primary human mesangial cells cultured in high

20   glucose was investigated by Northern Blotting as shown in Fig. 1.

In Fig. 1 the lanes represent the following:

Lane 1: RNA from mesangial cells exposed to 5 mM glucose;

25

Lane 2: RNA from mesangial cells exposed to 5 mM glucose and 25 mM mannitol;

Lane 3: RNA from mesangial cells exposed to 30 mM glucose for seven days.

5   A 2.4 kb band was detected following hybridisation with the CTGF probe. The relative amounts of CTGF mRNA as estimated by Phosphor Imager quantification are indicated in Fig. 2. All of the values were normalised to GAPDH levels and the results are representative of three independent experiments.

10

The results indicate that CTGF mRNA expression was between 2.5-3.3-fold higher in mesangial cells cultured in 30 mM glucose as compared with 5 mM glucose.

15   <u>b) Effect of CTGF on mesangial cell matrix production.</u>

To investigate the direct effects of CTGF up-regulation on matrix production, in particular the effect on collagens I and IV and fibronectin, mesangial cells were incubated with recombinant CTGF

20   protein.

Mesangial cells were serum starved for 24 hr in RPMI 1640 medium supplemented with 0.5 % fetal bovine serum (FBS) and then exposed to rhCTGF (8 ng/ml) (a generous gift from Dr. Gary

25   Grotendorst) for 24 hr (Kreisberg, J.I. and Ayo, S.H. (1993). *Kidney Int.* *43*, 109-113). Total RNA was extracted and chromosomal DNA was removed using DNase 1 (Gibco-BRL). Equal amounts of cDNA were

24

subsequently amplified by PCR using specific primers for GAPDH

(Gen/EMBL accession no. AJ005371, sense:

ACCACAGTCCATGCCATCAC (SEQ ID NO: 7); antisense:

TCCACCACCCTGTTGCTGTA (SEQ ID NO: 8), Collagen I

5   (Gen/EMBL accession no. X55525, sense:

GGTCTTCCTGGCTTAAAGGG (SEQ ID NO: 9); antisense:

GCTGGTCAGCCCTGTAGAAG (SEQ ID NO: 10)), Collagen IV

(Gen/EMBL accession no. M11315, sense:

CCAGGAGTTCCAGGATTTCA (SEQ ID NO: 11); antisense:

10   TTTTGGTCCCAGAAGGACAC (SEQ ID NO: 12) and fibronectin

(Gen/EMBL accession no. X02761, sense:

CGAAATCACAGCCAGTAG (SEQ ID NO. 13), antisense:

ATCACATCCACACGGTAG (SEQ ID NO: 14)).

15   Fig. 3 depicts ethidium-stained panels of a 2% (w/v) agarose gel

containing 10 µl of each PCR reaction after electrophoresis.

In Fig. 3 the lanes represent the following:

20   Lane 1: RT-PCR products from mesangial cells cultured in

RPMI 1640 and 0.5% FBS;

Lane 2: RT-PCR products from mesangial cells exposed to

rhCTGF (8 ng/ml) for 24 hr.

25

These results indicate that rhCTGF up-regulates mesangial cell collagens I and IV and fibronectin. These proteins typify matrix accumulation as seen in diabetic nephropathy.

5   CTGF is a member of a small family of highly homologous proteins termed the CCN family (for *CTGF* / fisp-12, *cef*10/cyr61 and *Nov*) (Bork, P (1993). *FEBS Letts. 327*, 125-130.). These peptides are characterised by conservation of 38 cysteine residues that constitute more than 10 % of the amino acid content. All members have signal

10   peptides and appear to be secreted *via* orthodox secretory pathways (Bradham, D.M., (1991) *J. Cell. Biol. 114*, 1285-1294). In the context of diabetic nephropathy, it is intriguing that CTGF which is up-regulated in the presence of ambient glucose, in turn, up-regulates the production of extracellular matrix (ECM). These data demonstrate the potential of

15   CTGF as a stimulus for increased ECM synthesis and mesangial expansion in diabetic nephropathy.

Example 3

20   Enhanced CTGF expression in renal cortex and isolated glomeruli of rats with STZ-induced diabetic nephropathy.

To assess CTGF expression in diabetic nephropathy *in vivo,* CTGF mRNA levels were measured in RNA isolated from the cortex of

25   rats with STZ-induced diabetes mellitus. To this end, PCR primers for rat CTGF were designed from the sequence of the mouse CTGF homologue, fisp12 (Genbank/EMBL accession no. M70642, sense:

CTAAGACCTGTGGAATGGGC (SEQ ID NO: 15); antisense:
CTCAAAGATGTCATTGTCCCC (SEQ ID NO: 16)) (Ryseck, R.P.,
(1991) *Cell Growth Differ.* 2, 225-233).

5   RT-PCR was performed on total RNA extracted from renal
cortex of STZ-diabetic rats and age matched controls. The sequence of
the rat CTGF transcript was 94 % identical at the nucleotide level (Fig.
4) and 99 % identical at the amino acid level (Fig. 5) to the mouse
CTGF homologue fisp12 (bases 783-1123, accession no. M70642).
10   Nucleotides that differ between the two species are given in upper case
and the single different amino acid is in bold.

        Induction of CTGF mRNA was observed in the renal cortex of
rats with STZ-induced diabetic nephropathy at four months after
15   administration of STZ, coincident with mesangial expansion and
proteinuria as shown in Fig. 6 and data not shown.

        Fig. 6 depicts ethidium-stained panels of a 2 % (w/v) agarose gel
containing 10 μl of each PCR reaction after electrophoresis. CTGF and
20   GAPDH mRNA levels were analysed in total RNA purified from 2
diabetic animals with established nephropathy after four months of
diabetes (lanes 1 and 2) and two age matched control animals (lanes 3
and 4).

25   CTGF expression was further localized to glomeruli by RT-PCR
analysis of RNA extracted from glomeruli isolated by differential
sieving from the renal cortex of rats with STZ-induced diabetic

nephropathy. Glomerular levels of CTGF mRNA were increased by 2.5-fold and 1.6-fold after two months and four months of diabetes, respectively, by comparison with age and sex-matched controls. The significance of these observations is further supported by a recent report demonstrating CTGF expression in a screen of human renal diseases including diabetic nephropathy (Ito, Y., *et al.* (1998) *Kidney Int. 53*, 853-861).

<u>Example 4</u>

<u>Induction of mesangial cell CTGF expression by high glucose involves TGF-β1 dependent and independent pathways.</u>

It has been shown that TGF-β1 is a stimulus for mesangial matrix accumulation in diabetic nephropathy. In our experimental model as described in Example 1, high glucose concentrations provoked induction of TGF-β1 mRNA expression in cultured human mesangial cells over the same temporal framework as CTGF expression (data not shown).

To assess the role of TGF-β1 as a stimulus for CTGF expression in response to high glucose, cells were incubated in either 5 mM glucose or 30 mM glucose plus 1 μl/ml anti-TGF-β1 antibody for seven days with three changes of medium. Cells were serum starved for 24 hr in RPMI 1640 and 0.5% FBS. 10 ng/ml TGF-β1 (Calbiochem) or 10 ng/ml TGF-β1 preadsorbed with 1 μg/ml neutralising anti-TGF-β1 polyclonal antibody were subsequently added for 24 hr.

The role of PKC on CTGF expression in response to high glucose was investigated by culturing the mesangial cells in either 5 mM, 30 mM glucose or 30 mM glucose and the PKC inhibitor GF 102903X.

5

Fig. 7 is an autoradiograph of CTGF mRNA levels analysed by Northern Blot and depicts the results obtained when mesangial cells were exposed to TGF-β1 (10 ng/ml) for 24 hr in the presence (lane 3) and absence (lane 2) of anti-TGF-β1 neutralising antibody (1 μg/ml).

10   Cells cultured in RPMI 1640 and 0.5 % FBS for 24 hr served as control (lane 1). A 2.4 kb band was detected following hybridisation to the CTGF probe. The blot was stripped and reprobed with GAPDH. The relative amount of CTGF mRNA as estimated by Phosphor Imager quantification (Fig. 8). Values were normalised to GAPDH levels and

15   the results are representative of two independent experiments.

These results indicate that TGF-β1 is a potent inducer of increased CTGF mRNA levels under these conditions. This effect was inhibited by the addition of a neutralising anti-TGF-β1 antibody as

20   depicted in Fig. 7.

Fig. 9 is an autoradiograph of CTGF mRNA levels analysed by Northern Blot and depicts the results obtained when mesangial cells were exposed to 5 mM glucose (lane 1), 30 mM glucose (lane 2) and 30

25   mM glucose in the presence of anti-TGF-β1 neutralising antibodies (1 μg/ml) (lane 3) for seven days. A 2.4 kb band was detected following hybridisation to the CTGF probe. The blot was stripped and probed with

GAPDH. The relative amount of CTGF mRNA as estimated by Phosphor Imager quantification (Fig. 10). Values were normalised to GAPDH levels.

5   The neutralising anti-TGF-β1 antibody partially attenuated the glucose-induced increase in CTGF transcript level in mesangial cells grown in 30 mM glucose for 7 days (Fig. 9), suggesting that high glucose triggers mesangial cell CTGF expression through TGF-β1-dependent and independent pathways.

10

         Fig. 11 is an autoradiograph of CTGF mRNA levels analysed by Northern Blot and depicts the results obtained when mesangial cells were exposed to 5 mM glucose (lane 1), 30 mM glucose (lane 2) and 30 mM glucose in PKC inhibitor GF102903X (10 µM) (lane 3) for four

15   days. A 2.4 kb band was detected following hybridisation to the CTGF probe. The blot was stripped and probed with GAPDH. The relative amount of CTGF mRNA as estimated by Phosphor Imager quantification (Fig. 12). Values were normalised to GAPDH levels.

20   Whereas the PKC inhibitor GF102903X was without effect on TGF-β1-induced CTGF expression in our system (data not shown), this compound afforded partial inhibition of high glucose-induced CTGF expression (Fig. 11).

25   CTGF shares some of the biological actions of TGF-β1 such as stimulation of cell proliferation and extracellular matrix protein synthesis in fibroblasts. When considered in this context, our results

suggest that TGF-β1 may promote mesangial matrix production, in part, by inducing CTGF synthesis. TGF-β1 has a complex profile of biological activities that includes pro-inflammatory, pro-fibrotic and anti-inflammatory effects. By targeting CTGF it may be possible to

5   attenuate the sclerosis-inducing effects of TGF-β1 while preserving its more desirable anti-inflammatory activities.

<u>Example 5</u>

10   <u>Further characterisation of IHG-2</u>

     IHG-2 is a mesangial cell gene which we have identified as being induced in human mesangial cells by high extracellular glucose as described in Example 1. To further characterise this gene, IHG-2 was

15   searched against the dbEST using the BLAST algorithm. This search identified a clone that was 94 % identical to ESTAA071138, clone no: 530117 3'. The sequence for the 5' end of this clone was also in the database, which again identified multiple ESTs. These ESTs showed homology with the 3' untranslated region (UTR) of a rat cDNA clone

20   known as drm/Gremlin. As indicated above, gremlin/drm, together with DAN and cerberus, are members of the cysteine knot super-family which includes TGFβ and bone morphogenetic protein (BMP). A second EST W48852, clone no:324951 3', was identified from the IHG-2 BLAST. The 5' end of this clone, EST W48619, was also searched

25   against the database, from which EST AA373348 was obtained. This clone showed homology with the drm 3' UTR, approximately 500 bp from the open reading frame (ORF). Thus, it was possible to make a

direct link from IHG-2 to within 500 bp of the ORF of drm/gremlin. Therefore, by establishing a link between EST AA37348 and the ORF of drm/gremlin, it was confirmed that IHG-2 is part of the 3' UTR of this gene. Primers were designed to recognise the ORF, IHG-2, and the EST

5   clone AA373348. An initial PCR using primers corresponding to the start site of the gremlin/drm gene together with a primer within the IHG-2 clone would give a predicted product of approximately 2.5 kb. This product was nested with primers corresponding to the 3' end of the ORF of gremlin and the EST clone AA373348, generating a product of

10   approximately 500 bp, thus verifying that this EST is in the UTR of the human drm/gremlin gene. Therefore, IHG-2 was found to be part of the drm/gremlin gene, which was not previously known (Fig. 13).

Example 6

15

Use of cloning *in-silico* coupled with PCR to demonstrate that IHG-2 is part of the 3' untranslated region of gremlin

In Example 5 we describe the identification of a transcript, IHG-2, the

20   sequence of which did not show homology against the cumulative database of characterised sequences using the BLAST algorithm.

Bioinformatic analysis was carried out as follows:

25   Database searching and alignments were performed at the National Center for Biotechnology Information (NCBI) Bethesda, Maryland, U.S.A. using the Basic Local Alignment Search Tool

Algorithm (BLAST) (Altschul, S.F., *et al.* (1997) *Nucleic Acids Res.* 25, 3389-3402). The Non Redundant (nr) and the Expressed Sequence Tag (EST) databases were sourced. Contiguous sequences were generated using Fragment Assembly, a program within the Genetics Computer
5   Group Inc. package. UniBlast (Guffanti, A., and Simon, G., *Trends in Genetics*, 14, 293) was used to identify homologous clusters within the UniGene database and to verify the consensus sequence derived from ESTs. Chromosomal localization data were obtained from the UniGene and Online Mendelian Inheritance in Man (OMIM) databases.
10

         To further characterise the sequence, the sequence of IHG was searched against the EST database where a number of matches were obtained. Each of these matches was, in turn, searched against the nr database at NCBI. Four ESTs, namely W52686, N28395, H80042, and
15   W47324, showed low homology to the 3' UT region of the rat gene drm referred to in Example 5. The ORF of the human homologue of this gene, gremlin, was also in the database.

         To generate a link between the ORF of gremlin and IHG-2,
20   successive BLAST searches were used to identify overlapping sequences in the EST database.

         However, it was not possible to directly link IHG-2 to the ORF of gremlin with sequences within the EST database. Therefore, RNA
25   isolated from human mesangial cells was reverse transcribed with a primer that recognises IHG-2. PCR analyses were performed spanning the regions shown in Fig. 13.

In this graphical representation of a BLAST output from the
EST database, the thick bar of 4 kb represents the final composite
sequence of the gremlin gene. Each of the other bars represents an
5   individual sequence, in the EST database, that were assembled, where
possible, into continuous sequences, and demonstrates how cloning *in-
silico* was used to generate the contiguous sequence. The region of no
EST overlap was generated by reverse transcription of human mesangial
mRNA with a complementary primer to IHG-2. PCR was performed
10   spanning the regions indicated (by arrows), and the resulting products
were sequenced, allowing a contiguous cDNA of 4049 bp to be
generated which included the open reading frame of gremlin and IHG-2.

Human gremlin and rat drm cDNAs were compared and Fig. 14
15   shows a graphical representation of an alignment between rat drm and
human gremlin together with the region corresponding to IHG-2 using
the BLAST algorithm. Sequence homology was found to be high in the
coding region of the cDNA; however, there are only small regions of
homology within the 3' UT region. This explains why IHG-2 did not
20   identify drm in a BLAST search, but a match to drm, and thus gremlin,
was obtained by examining EST sequences further. The lack of
homology between rat drm and human gremlin probably results from
decreased selective pressure on the 3' UT region of gene homologues to
remain the same between species.
25

Fig. 15 shows the final sequence of human mesangial cell
gremlin, indicating the ORF and the region corresponding to IHG-2

34

(GenBank accession no: AF110137). Shown are the 5' and 3' UT sequence, and the open reading frame (with translation). The boxed region corresponds to the location of IHG-2. At the time of submission, this sequence matched 136 separate EST entries in the EST database. Of

5   these entries, 23.5% were derived from fibroblast libraries; 30% were from bone tissue libraries; and 34% were derived from tumor related libraries. The sequence was also searched against the UniGene database using the UniBlast program. This identified 4 UniGene clusters, Hs.214148, Hs.40098, Hs.114330, and Hs.239507. Two of these

10   clusters, Hs.40098, and Hs.239507, have been mapped to intervals D15S118-144 and D15S144-165 on chromosome 15, respectively. Secretory granule neuroendocrine protein 1 and the alpha polypeptide of the nicotinic cholinergic receptor have been mapped to either side of these clusters and have also been mapped more specifically to the

15   15q11-15 interval. Analysis of the OMIM database reveals that the formin gene, which was recently shown to induce gremlin expression in the developing limb bud (Zuniga, A., et al. (1999) supra), is also localised to this interval. Diabetes mellitus with multiple epiphyseal dysplasia, or Wolcott-Rallison syndrome, is localised to the 15q11-12

20   interval (Stewart, F.J., et al., (1996) Clin.Genet. 49, 152-5). In the epiphyseal growth plate, immunohistochemical studies have revealed that BMP-2 and 4 are expressed in proliferating and maturing chrondocytes, suggesting that BMP and its receptors play roles in the multi-step cascade of enchondral ossification (Yazaki, Y., et al., (1998)

25   Anticancer Res. 18, 2339-44). Regulation of gremlin expression may have implications in both of the disease states associated with Wolcott-Rallison syndrome.

Example 7

Induction of mesangial cell gremlin expression *in vitro* by high glucose and cyclic mechanical strain induce

A) Primary cultures of human mesangial cells were propagated as described in Example 1, except that the medium was supplemented with 5% FBS. Treatment of cultures with glucose was carried out as described in Example 1. Mesangial cells were exposed to either 5m M glucose or 30mM glucose for seven days.

Northern Blot analysis as described further below was performed on RNA extracted from mesangial cells grown in either 5 mM ('normal') or 30 mM ('high') glucose using the ORF of gremlin and IHG-2 probes. Both probes detected a 2-fold increase in gene expression under high glucose conditions as depicted in Fig. 16.

All subsequent northern analysis was performed using the ORF of gremlin as a probe.

Northern Blots were performed using formaldehyde denaturation according to standard protocols and quantitated using a phosphorimager (Biorad). PCR products used to generate the probes for northern analysis were amplified using primers for the open reading frame (ORF) of gremlin (sense: ATGAGCCGCACAGCCTACAC (SEQ ID NO: 17); antisense TTAATCCAAATCGATGGATATGC (SEQ ID NO: 18)), and

for IHG-2 (sense: CTCAGCCTCCTAGCCAAGTCC (SEQ ID NO 19);
antisense: GTATTGTCCACATTCTCCAAC (SEQ ID NO: 20)).
Fibronectin and GAPDH probes were generated as described in
Example 2

5   Specific primers were used to amplify gremlin/IHG-2 (external sense:
ATGAGCCGCACAGCCTACAC (SEQ ID NO: 21); external antisense:
GTATTGTCCACATTCTCCAAC (SEQ ID NO: 22); internal sense:
GAGAGTCACACGTGTGAAGC (SEQ ID NO: 23); internal antisense:
AGGAGGATGCAAGCACAGG (SEQ ID NO: 24), BMP-2 (external

10   sense: CGCGGATCCTGCTTCTTAGACGGACTGCG (SEQ ID NO:
25); external antisense: TTTGCTGTACTAGCGACACC (SEQ ID NO:
26); internal sense: CAAGATGAACACAGCTGG (SEQ ID NO 27)),
and GCTCAGGATACTCAAGAC (SEQ ID NO: 28)). RT-PCR was
carried out as reported as described in Example 3.

15

The results are shown in Figs. 16 and 17.

In Figs. 16 and 17, lane 1 corresponds to the mesangial cells
exposed to 5 mM glucose and lane 2 corresponds to the mesangial cells
20   exposed to 30 mM glucose.

Fig. 16 is an autoradiograph of IHG-2 (1), gremlin (2),
fibronectin (3) and GAPDH (4) mRNA levels analysed by Northern
Blot. Two bands of approximately 4.4 kb and 4.6 kb were detected
25   following hybridisation to the gremlin and IHG-2 probes.

37

Fig. 17 depicts relative mRNA levels as estimated by Phosphor
Imager quantification. Values were normalised to GAPDH levels. The
results are representative of three independent experiments.

5   B) Glomerular hypertension is an independent risk factor for the
development of glomerulosclerosis in diabetes mellitus (Brenner, B.M.,
et al. (1982) N. Engl. J. Med. 307, 652-9). To model the effects of
glomerular hypertension on mesangial cell gremlin expression in the
present study, mesangial cells were propagated under conditions of
10   cyclic mechanical strain for 24 and 48 h in the Flexercell ^{TM} System

For the application of mechanical cyclic stretch, primary human
mesangial cells were seeded on either flexible or rigid based, elastin
coated six-well plates (Flex I and Flex II plates, Flex Cell^{TM} Int,
15   Hillsborough, NH, USA). Cells were grown to 90% confluency, then
serum restricted in Clonetics^{TM} Mesangial Basal Medium supplemented
with 0.5% fetal calf serum. Cells cultured on flexible plates were
subjected to repeated cycles of computer-controlled, vacuum-driven
mechanical stretch and relaxation using the Flexercell Strain Unit FX-
20   2000 (Flexercell^{TM}). Cells were alternately stretched and relaxed at 0.5
sec intervals (60 cycles/min) for either 24 or 48 h. The applied vacuum
achieved a 17% elongation of the outer annulus of the culture plates. All
experiments were carried out at 37^0 C and 5% CO_2 in a humidified
incubator.

25

38

The results were obtained from three diabetic rats, 14 weeks following onset of diabetes and from three age matched controls.

5   The model used perturbs mesangial cell matrix production and metabolism in a manner similar to that observed in diabetic glomerulosclerosis *in vivo*. Mesangial cell gremlin mRNA levels were significantly enhanced under conditions of mechanical strain, in parallel with increased fibronectin mRNA expression as shown in Figs. 18 and 19.

10

Referring to Figs. 18 and 19 mesangial cells in culture were grown under static conditions (lane 1) or during exposure to cyclic stretch for 24 h (lane 2) or 48 h (lane 3) using the Flexercell ™ System.

15   Fig. 18 is an autoradiograph of gremlin(1), fibronectin(2) and GAPDH(3) mRNA analysed by Northern Blot.

In Fig. 18 the lanes represent the following:

Lane 1: Mesangial cells grown in culture under static conditions;

20   Lane 2: Mesangial cells grown in culture during exposure to cyclic stretch for 24 h; and

Lane 3: Mesangial cells grown in culture during exposure to cyclic stretch for 48 h.

25   Fig. 19 depicts relative mRNA levels as estimated by Phosphor Imager quantification. Values were normalised to GAPDH levels.

To assess gremlin expression in diabetic nephropathy *in vivo*, gremlin mRNA levels were measured by Northern Blot analysis with RNA isolated from the renal cortex of control rats and from diabetic rats 14 weeks after induction of diabetes mellitus by streptozotocin (STZ).

5　　In keeping with the *in vitro* experiments reported above, gremlin mRNA levels were increased in kidneys from diabetic rats, coincident with proteinuria and histologic evidence of diabetic nephropathy (data not shown).

10　　Nine Male Munich-Wistar uninephrectomized rats were rendered diabetic with streptozotocin, and the RNA extracted as described in Example 3.

The results are shown in Figs. 20 and 21, which show gremlin

15　　mRNA levels in renal cortex of STZ-diabetic rats.

Fig. 20 is an autoradiograph of gremlin mRNA levels in the kidney cortex of a STZ-diabetic rat (lane 2) and an age matched control (lane 1) analysed by Northern Blot. A band of approximately 4.4kb was

20　　detected following hybridisation to the gremlin probe.

Fig. 21 depicts relative mRNA levels as estimated by Phosphor Imager quantification. Values were normalised to GAPDH levels.

25

40

Example 8

Regulation of mesangial cell gremlin expression by high glucose: evidence for involvement of TG-β1

As indicated above, both high ambient glucose concentrations and cyclic mechanical strain provoke TGF-β1 production by mesangial cells *in vitro* and TGF-β1 appears to be a major stimulus for mesangial matrix accumulation in diabetic glomeruli *in vivo*. To probe the mechanism by which high glucose triggers gremlin expression, primary human mesangial cells were propagated in 5 mM or 30 mM glucose in the presence and absence of anti-TGF-β1 neutralising antibody (1 µg/ml). Treatment of cultures with glucose and anti-TGF-β1 were as described in Example 1 and Example 4, respectively. To assess the role of TGF-β1 as a stimulus for gremlin expression, cells were serum restricted for 24 h in MCDB131 and 0.5% FBS and subsequently treated with 10ng/ml TGF-β1. MCDB131 is a specialised medium for the growth of mesangial cells and is obtained from Clonetics.

Initial studies had indicated that TGF-β1 neuturalizing antibody (data not shown) blunted glucose-triggered gremlin expression and therefore the ability of TGF-β1 to alter gremlin expression was investigated. The results are shown in Figs. 22 and 23.

The addition of exogenous human recombinant TGF-β1 (10 ng/ml, 24 h) to serum restricted (24 h) mesangial cells also augmented

41

gremlin mRNA levels, suggesting that high glucose enhances gremlin mRNA expression, at least in part, through its ability to stimulate TGF-$\beta 1$ expression. In aggregate, these observations suggest the presence of a novel autocrine loop through which TGF-$\beta 1$ induces gremlin gene

5   expression and may thereby regulate the activity of mesangial-derived BMPs as hereinafter described.

It was found that gremlin expression in response to high glucose (30 mM, 7 days) was reduced in the presence of anti-TBF-$\beta 1$ antibody

10   (data not shown). To further probe the role of TGB-$\beta 1$ as a modulator of gremlin expression, mesangial cells were exposed to TGF-$\beta 1$ (10 ng/ml) for 24 h (lane 2). Cells cultured in MCDB131 and 0.5% FBS for 24 h served as a control (lane 1).

15   Fig. 22 is an autoradiograph of gremlin (1), fibronectin (2) and GAPDH (3) mRNA levels analysed by Northern Blot.

Fig. 23 shows relative mRNA levels as estimated by Phosphor Imager quantification. Values were normalised to GAPDH levels.

42

Example 9

High glucose stress induces BMP-2, but not BMP-4 expression in mesangial cells.

As indicated above gremlin is a putative antagonist of BMP-2 and BMP-4. Specifically, gremlin has been recently reported to form heterodimers with BMPs and thereby antagonise BMP signalling (Hsu, D.R., et al. (1998) *supra*). In the present study, RT-PCR was employed as an initial assessment of mesangial cell BMP expression.

As an initial assessment of the relationship between gremlin expression and BMP expression, RNA was isolated from mesangial cells grown for 7 days in either 5mM or 30mM glucose. Following reverse transcription with random primers, a primary PCR of the ORF of BMP-2 was performed. This product, which was undetectable on an ethidium stained agarose gel after 30 cycles, was nested to give a predicted product of 446 bp. PCR analysis with BMP-4 and GAPDH specific primers gave predicted products of 378 bp and 452 bp respectively.

Fig. 24 depicts representative reactions of 4 independent experiments. 10uL of each PCR reaction was run on 1 % ethidium bromide stained agarose gels.

Whereas little or no BMP-2 mRNA was detected in mesangial cells propagated in 5 mM glucose, a marked induction of BMP-2 expression was observed in cells cultured in 30 mM glucose as shown in Fig. 24.

5

In contrast, BMP-4 expression levels were relatively unchanged. Interestingly, whereas BMP-2 does not stimulate fibronectin expression in mesangial cells *in vitro*, BMP-2 has been recently shown to block mesangial cell proliferation triggered by epidermal growth factor and

10   platelet-derived growth factor (Ghosh Choundhury, G., *et al.*, (1999) *J. Biol. Chem. 274*, 10897-902; Ghosh Choudhury, G., *et al.*, (1999) *Biochem. Biophys. Res. Commun. 258*, 490-6). Our results raise the possibility that TGF-β1 stimulated expression of gremlin may contribute to mesangial cell proliferative responses in this setting. The influence of

15   gremlin on cell proliferation appears complex, however, and may vary markedly depending on the cell-type and proliferative stimulus. In contrast to the aforementioned potentially pro-proliferative actions, over-expression of the gremlin homologue, drm, causes apoptosis in fibroblasts by an ERK mediated pathway, while cells transformed with

20   oncogenes such as v-mos show suppressed drm expression (Topol, L.Z., *et al.*, (1997) *Mol. Cell. Biol.* 17, 4801-10). Similarly, over-expression of DAN, another cysteine knot super-family member with homology to drm/gremlin, retards fibroblast entry into S phase (Ozaki, T., *et al.*, (1995) *Cancer Res. 55*, 895-900).

25

In summary, our results demonstrate that the DAN family member gremlin is induced in diabetic nephropathy *in vivo* and implicate both metabolic and hemodynamic stress as stimuli for gremlin expression.

5   The findings that high glucose-triggered gremlin expression is mimicked by addition of exogenous TGF-β1, blunted by anti-TGF-β1 neutralising antibody, and occurs in association with induction of mesangial cell BMP-2 suggests the presence of a novel autocrine loop which may limit the bioactivity of TGF-β1 superfamily members and

10   modulate mesangial cell proliferation within the diabetic mesangium. The further elucidation of the functional interactions of the DAN family of secreted proteins, such as gremlin, with TGF-β1 superfamily members may shed light on the complex multi-pronged molecular events that perturb cell proliferation and matrix production in diabetic

15   glomerulosclerosis.

Claims: -

1.   A method for identifying a gene having a role in the presentation
of diabetic nephropathy, which method comprises culturing mesangial
cells in a medium in the presence of a concentration of glucose
sufficient to induce differential expression of a gene susceptible to such
differential expression and identifying the gene so induced.

2.   A method according to Claim 1, wherein the mesangial cells are
cultured in the presence of a concentration of glucose sufficient to
induce up-regulation of a gene susceptible to such up-regulation.

3.   A method according to Claim 1 or 2, wherein the concentration
of glucose is greater than 5 mM.

4.   A method according to any preceding claim, wherein the
mesangial cells are subjected to mechanical strain.

5.   A method according to any preceding claim, wherein
transforming growth factor $\beta 1$ (TGF-$\beta 1$) is added to the culture
medium.

6.   A method according to any preceding claim, wherein the
differentially expressed gene is identified by suppression subtractive
hybridisation.

7.   A method according to any one of Claims 1-6, wherein the possibility of differential expression due to hyperosmolarity is excluded.

8.   A method according to any one of Claims 1-7, wherein the gene so differentially expressed is a gene which includes a sequence selected from:

1) SEQ ID NOS: 1-3;

2) SEQ ID NO: 4;

3) SEQ ID NO: 5; and

4) SEQ ID NO: 6.

9.   Use of a gene identified by a method according to any one of Claims 1-8, as a diagnostic marker for the progression and presentation of diabetic nephropathy.

10.   Use of a gene identified by a method according to any of Claims 1-8, as an index of disease activity and the rate of progression of diabetic nephropathy.

11.   Use of a gene identified by a method according to any of Claims 1-8, as a basis for identifying drugs for use in the prevention and/or therapy of diabetic nephropathy.

12.   A gene containing a sequence selected from any one of
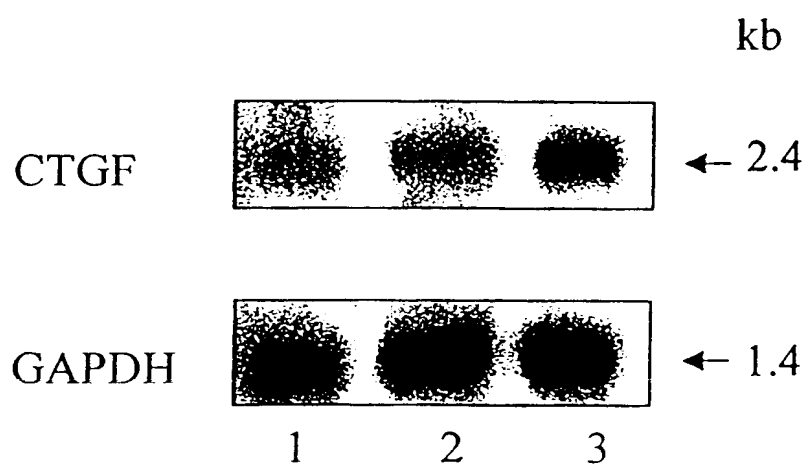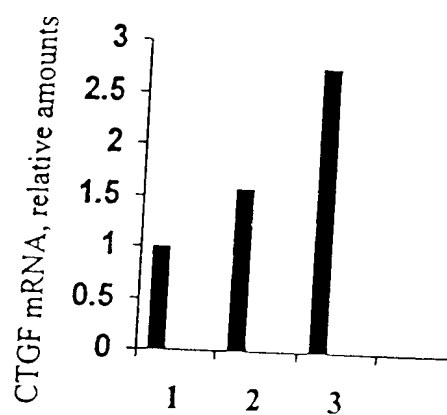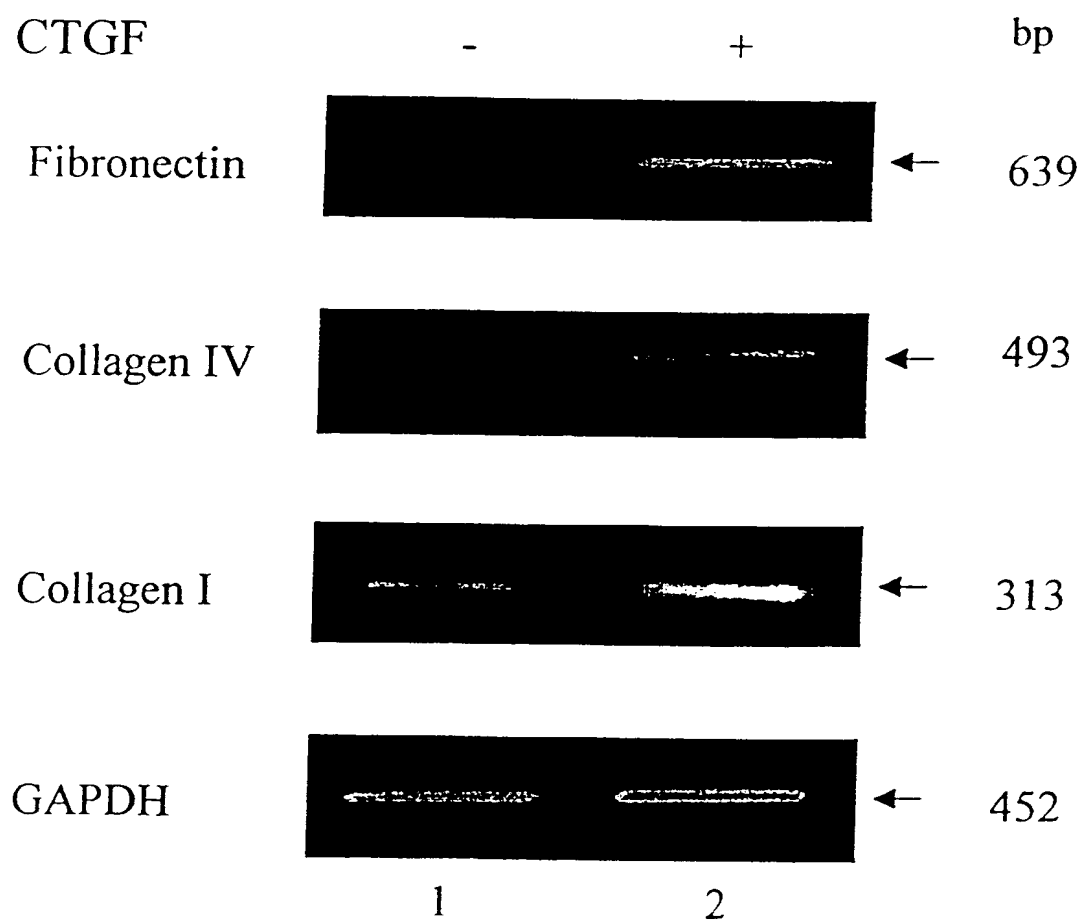sequences 1-3, 5 and 6 according to Claim 8.

5

1/24

Fig. 1

Fig. 2

3/24

Fig. 3

4/24

```
rat:    1   atctccacccgGgttaccaatgacaatacTttctgcagGctggagaagcagagTcgTctc 60
            !!!!!!!!!!!! !!!!!!!!!!!!!!!!!! !!!!!!!! !!!!!!!!!!!!!! !! !!!
mouse: 783  atctccacccgagttaccaatgacaataccttctgcagactggagaagcagagccgcctc 842


rat:   61   tgcatggtcaggccctgTgaagctgacctAgaggaaaacattaagaagggcaaaaagtgc 120
            !!!!!!!!!!!!!!!!! !!!!!!!!!!!! !!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
mouse: 843  tgcatggtcaggccctgcgaagctgacctggaggaaaacattaagaagggcaaaaagtgc 902


rat:  121   atccggacGcctaaaatTgccaagcctgtcaagtttgagctttctggctgcaccagtgtg 180
            !!!!!!!! !!!!!!!! !!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
mouse: 903  atccggacacctaaaatcgccaagcctgtcaagtttgagctttctggctgcaccagtgtg 962


rat:  181   aagacCtacCgggctaagttctgTggggtgtgcacGgacggccgctgctgcacaccgcac 240
            !!!!! !!! !!!!!!!!!!!!!! !!!!!!!!!! !!!!!!!!!!!!!!!!!!!!!!!!!!
mouse: 963  aagacatacagggctaagttctgcggggtgtgcacagacggccgctgctgcacaccgcac 1022


rat:  241   agaaccaccacActgccggtggagttcaaGtgcccccCatggcgaAatcatgaaaaagaac 300
            !!!!!!!!!!! !!!!! !!!!!!!!!! !!!!!! !!!!!!! !!!!!!!!!!!!!
mouse: 1023 agaaccaccactctgccagtggagttcaaatgccccgatggcgagatcatgaaaaagaat 1082


rat:  301   atgatgttcatcaagacctgtgcctgccattacaactgtcc 341     (SEQ ID NO: 29)
            !!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
mouse: 1083 atgatgttcatcaagacctgtgcctgccattacaactgtcc 112     (SEQ ID NO: 30)
```

Fig. 4

5/24

```
rat:     1   ISTRVTNDNTFCRLEKQSRLCMVRPCEADLEENIKKGKKCIRTPKIAKPVKFELSGCTSV 180
mouse: 216   ISTRVTNDNTFCRLEKQSRLCMVRPCEADLEENIKKGKKCIRTPKIAKPVKFELSGCTSV 275

rat    181   KTYRAKFCGVCTDGRCCTPHRTTTLPVEFKCPHGEIMKKNMMFIKTCACHYNC 339    (SEQ ID NO: 31)
mouse: 276   KTYRAKFCGVCTDGRCCTPHRTTTLPVEFKCPDGEIMKKNMMFIKTCACHYNC 328    (SEQ ID NO: 32)
```
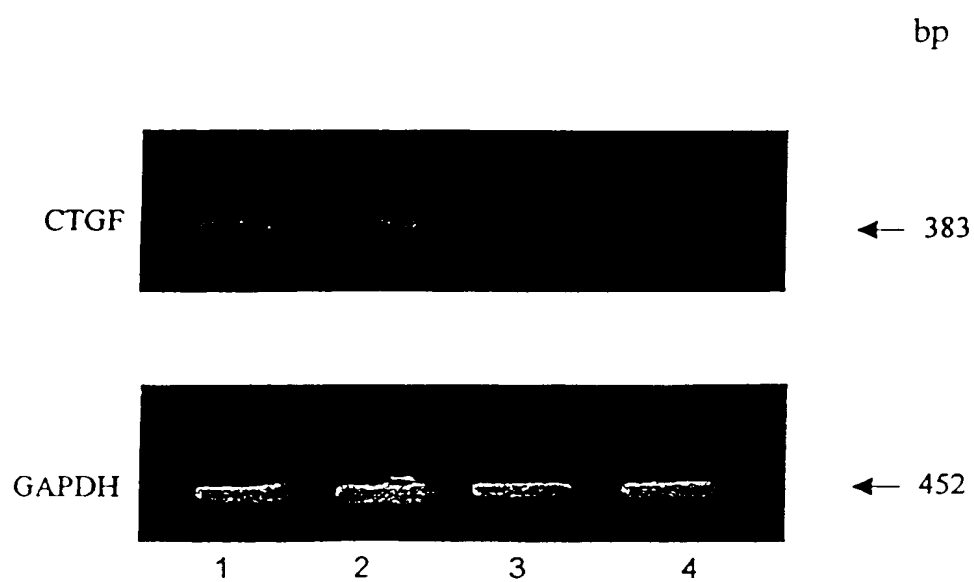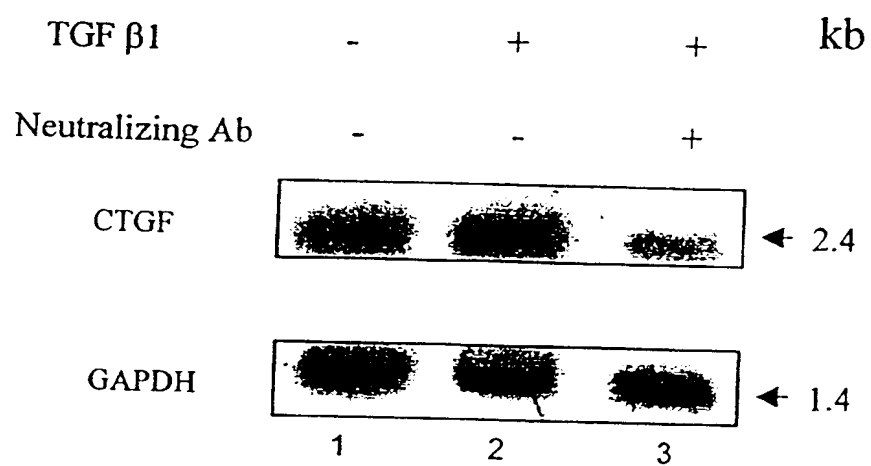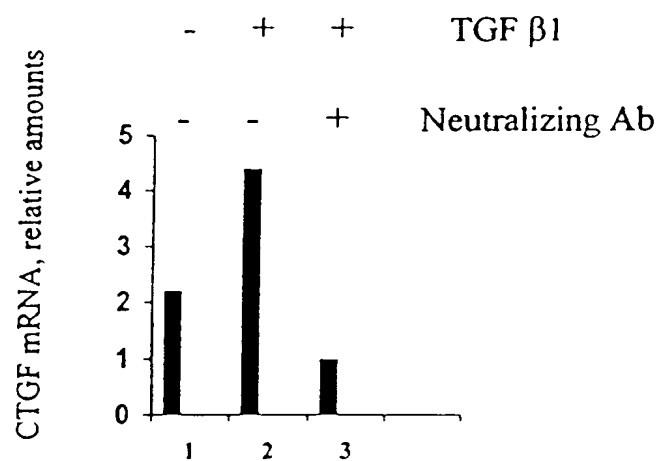
Fig. 5

Fig. 6
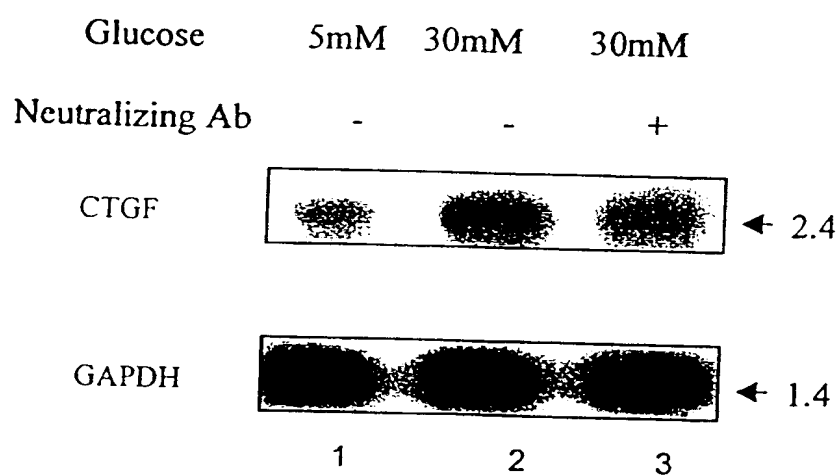
7/24

Fig. 7

8/24

Fig. 8

9/24

Glucose　　　　5mM　30mM　　　30mM

Neutralizing Ab　　　-　　　　-　　　　+

CTGF　　　　　　　　　　　　　　　　　　　　← 2.4
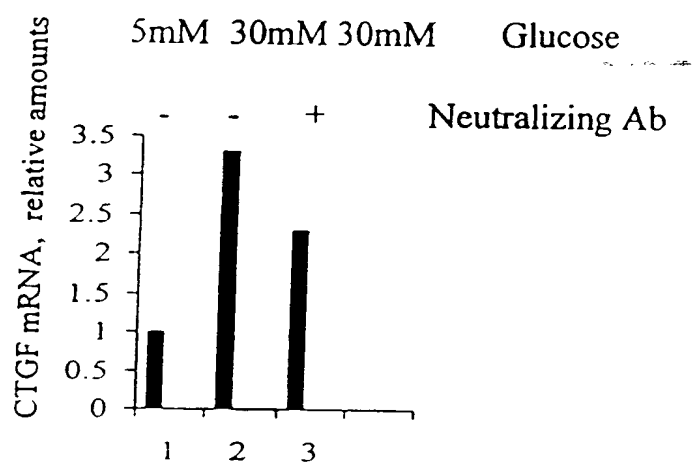
GAPDH　　　　　　　　　　　　　　　　　　　← 1.4

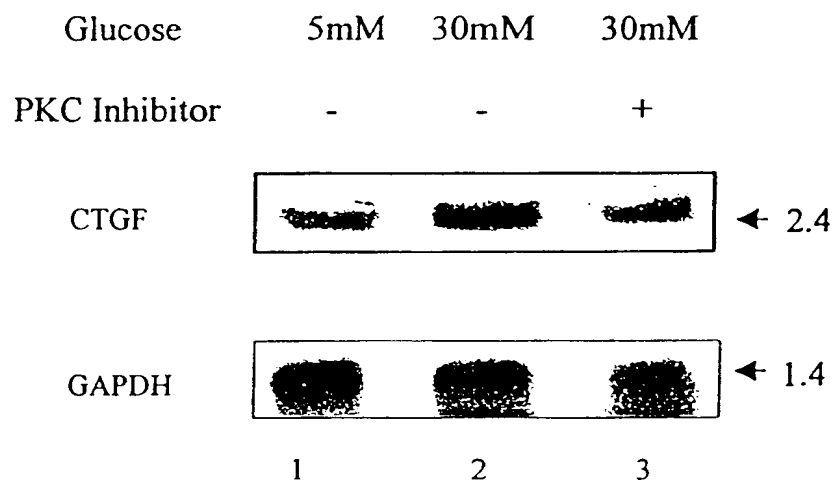　　　　　　　1　　　　　2　　　　　3

Fig. 9

Fig. 10

11/24

Fig. 11

12/24

Fig. 12

13/24

Fig. 13

Fig. 14
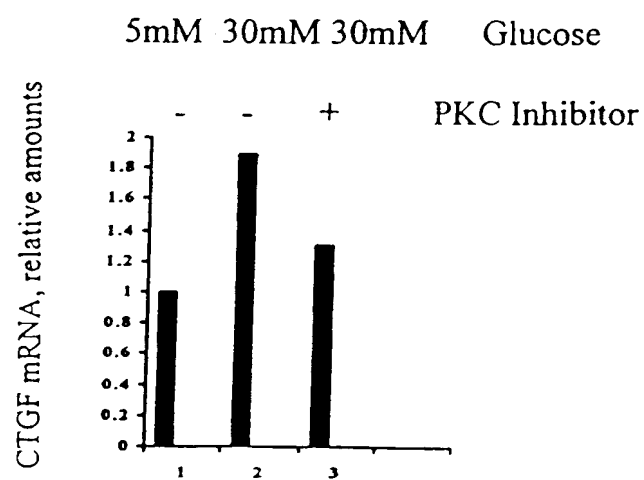
```
1      GCGGCCGCACTCAGCGCCACGCGTCGAAAGCGCAGGCCCCGAGGACCCGCCGCACTGACAGTATGAGCCGCACAG
                                                                      M  S  R  T  A
76     CCTACACGGTGGGAGCCCTGCTTCTCCTCTTGGGGACCCTGCTGCCGGCTGCTGAAGGGAAAAAGAAAGGGTCCC
        Y  T  V  G  A  L  L  L  L  L  G  T  L  L  P  A  A  E  G  K  K  K  G  S  Q
151    AAGGTGCCATCCCCCCGCCAGACAAGGCCCAGCACAATGACTCAGAGCAGACTCAGTCGCCCCAGCAGCCTGGCT
         G  A  I  P  P  P  D  K  A  Q  H  N  D  S  E  Q  T  Q  S  P  Q  Q  P  G  S
226    CCAGGAACCGGGGGCGGGGCCAAGGGCGGGGCACTGCCATGCCCCGGGGAGGAGGTGCTGGAGTCCAGCCAAGAGG
        R  N  R  G  R  G  Q  G  R  G  T  A  M  P  G  E  E  V  L  E  S  S  Q  E  A
301    CCCTGCATGTGACGGAGCGCAAATACCTGAAGCGAGACTGGTGCAAAACCCAGCCGCTTAAGCAGACCATCCACG
        L  H  V  T  E  R  K  Y  L  K  R  D  W  C  K  T  Q  P  L  K  Q  T  I  H  E
376    AGGAAGGCTGCAACAGTCGCACCATCATCAACCGCTTCTGTTACGGCCAGTGCAACTCTTTCTACATCCCCAGGC
        E  G  C  N  S  R  T  I  I  N  R  F  C  Y  G  Q  C  N  S  F  Y  I  P  R  H
451    ACATCCGGAAGGAGGAAGGTTCCTTTCAGTCCTGCTCCTTCTGCAAGCCCAAGAAATTCACTACCATGATGGTCA
         I  R  K  E  E  G  S  F  Q  S  C  S  F  C  K  P  K  K  F  T  T  M  M  V  T
526    CACTCAACTGCCCCTGAACTACAGCCACCTACCAAGAAGAAGAGAGTCACACGTGTGAAGCAGTGTCGTTGCATAT
        L  N  C  P  E  L  Q  P  P  T  K  K  K  R  V  T  R  V  K  Q  C  R  C  I  S
601    CCATCGATTTGGATTAAGCCAAATCCAGGTGCACCCAGCATGTCCTAGGAATGCAGCCCCAGGAAGTCCCAGACC
         I  D  L  D
676    TAAAACAACCAGATTCTTACTTGGCTTAAACCTAGAGGCCAGAAGAACCCCCAGCTGCCTCCTGGCAGGAGCCTG
751    CTTGTGCGTAGTTCGTGTGCATGAGTGTGGATGGGTGCCTGTGGGTGTTTTTAGACACCAGAGAAAACACAGTCT
826    CTGCTAGAGAGCACTCCCTATTTTGTAAACATATCTGCTTTAATGGGGATGTACCAGAAACCCACCTCACCCCGG
901    CTCACATCTAAAGGGGCGGGGCCGTGGTCTGGTTCTGACTTTGTGTTTTTGTGCCCTCCTGGGGACCAGAATCTC
976    CTTTCGGAATGAATGTTCATGGAAGAGGCTCCTCTGAGGGCAAGAGACCTGTTTTAGTGCTGCATTCGACATGGA
1051   AAAGTCCTTTTAACCTGTGCTTGCATCCTCCTTTCCTCCTCCTCACAATCCATCTCTTCTTAAGTTGATAGT
1126   GACTATGTCAGTCTAATCTCTTGTTTGCCAAGGTTCCTAAATTAATTCACTTAACCATGATGCAAATGTTTTTCA
1201   TTTTGTGAAGACCCTCCAGACTCTGGGAGAGGCTGGTGTGGGCAAGGACAAGCAGGATAGTGGAGTGAGAAAGGG
1276   AGGGTGGAGGGTGAGGCCAAATCAGGTCCAGCAAAAGTCAGTAGGGACATTGCAGAAGCTTGAAAGGCCAATACC
1351   AGAACACAGGCTGATGCTTCTGAGAAAGTCTTTTCCTAGTATTTAACAGAACCCAAGTGAACAGAGGAGAAATGA
1426   GATTGCCAGAAAGTGATTAACTTTGGCCGTTGCACTCTGCTCAAACCTAACACCAAACTGAAAACATAAATACTG
1501   ACCACTCCTATGTTCGGACCCAAGCAAGTTAGCTAAACCAAACCAACTCCTCTGCTTTGTCCCTCAGGTGGAAAA
1576   GAGAGGTAGTTTAGAACTCTCTGCATAGGGGTGGGAATTAATCAAAAACCKCAGAGGCTGAAATTCCTAATACCT
1651   TTCCTTTATCGTGGTTATAGTCAGCTCATTTCCATTCCACTATTTCCCATAATGCTTCTGAGAGCCACTAACTTG
1726   ATTGATAAAGATCCTGCCTCTGCTGAGTGTACCTGACAGTAAGTCTAAAGATGARAGAGTTTAGGGACTACTCTG
1801   TTTTAGCAAGATATATTKTGGGGGTCTTTTTGTTTTAACTATTGTCAGGAGATTGGGCTARAGAGAAGACGACGA
1876   GAGTAAGGaAATAAAGGGRATTGCCTCTGGCTAGAGAGTAAGTTAGGTGTTAATACCTGGTAGAAATGTAAGGGA
1951   TATGACCTCCCTTTCTTTATGTGCTCACTGAGGATCTGAGGGGACCCTGTTAGGAGAGCATAGCATCATGATGTA
2026   TTAGCTGTTCATCTGCTACTGGTTGGATGGACATAACTATTGTAACTATTCAGTATTTACTGGTAGGCACTGTCC
2101   TCTGATTAAACTTGGCCTACTGGCAATGGCTACTTAGGATTGATCTAAGGGCCAAAGTGCAGGGTGGGTGAACTT
2176   TATT GTACTTTGGATTTGGTTAACCTGTTTTCTTCAAGCCTGAGGTTTTATATACAAACTCCCTGAATACTCTTT
2251   TTGCCTTGTATCTTCTCAGCCTCCTAGCCAAGTCCTATGTAATATGGAAAACAAACACTGCAGACTTGAGATTCA
2326   GTTGCCGATCAAGGCTCTGGCATTCAGAGAACCCTTGCAACTCGAGAAGCTGTTTTTATTTCGTTTTTGTTTTGA
2401   TCCAGTGCTCTCCCATCTAACAACTAAACAGGAGCCATTTCAAGGCGGGAGATATTTTAAACACCCAAAATGTTG
2476   GGTCTGATTTTCAAACTTTTAAACTCACTACTGATGATTCTCACGCTAGGCGAATTTGTCCAAACACATAGTGTG
2551   TGTGTTTTGTATACACTGTATGACCCCCACCCCAAATCTTTGTATTGTCCACATTCTCC AACAATAAAGCACAGAG
2626   TGGATTTAATTAAGCACACAAATGCTAAGGCAGAATTTTGAGGGTGGGAGAGAAGAAAAGGGAAAGAAGCTGAAA
2701   ATGTAAAACCCACACCAGGGAGGGAAAAATGACATTCAGAACCAGCAAACACTGAATTTCTCTTGTTGTTTTAACTC
2776   TGCCACAAGAATGCAATTTCGTTAATGGAGATGACTTAAGTTGGCAGCAGTAATCTTCTTTTAGGAGCTTGTACC
2851   ACAGTCTTGCACATAAGTGCAGATTTGGCTCAAGTAAAGAGAATTTCCTCAACACTAACTTCACTGGGATAATCA
2926   GCAGCGTAACTACCCTAAAAGCATATCACTAGCCAAAGAGGGAAATATCTGTTCTTCTTACTGTGCCTATATTAA
3001   GACTAGTACAAATGTGGTGTGTCTTCCAACTTTCATTGAAAATGCCATATCTATACCATATTTTATTCGAGTCAC
3076   TGATGATGTAATGATATATTTTTTCATTATTATAGTAGAATATTTTTATGGCAAGATATTTGTGGTCTTGATCAT
3151   ACCTATTAAAATAATGCCAAACACCAAATATGAATTTTATGATGTACACTTTGTGCTTGGCATTAAAAGAAAAAA
3226   ACACACATCCTGGAAGTCTGTAAGTTGTTTTTTGTTACTGTAGGTCTTCAAAGTTAAGAGTGTAAGTGAAAAATC
3301   TGGAGGAGAGGATAATTTCCACTGTGTGGAATGTGAATAGTTAAATGAAAAGTTATGGTTATTTAATGTAATTAT
3376   TACTTCAAATCCTTTGGTCACTGTGATTTCAAGCATGTTTTCTTTTTCTCCTTTATATGACTTTCTCTGAGTTGG
3451   GCAAAGAAGAAGCTGACACACCGTATGTTGTTAGAGTCTTTTATCTGGTCAGGGGAAACAAAATCTTGACCCAGC
3526   TGAACATGTCTTCCTGAGTCAGTGCCTGAATCTTTATTTTTTAAATTGAATGTTCCTTAAAGGTTAACATTTCTA
3601   AAGCAATATTAAGAAAGACTTTAAATGTTATTTTGGAAGACTTACGATGCATGTATACAAACGAATAGCAGATAA
3676   TGATGACTAGTTCACACATAAAGTCCTTTTAAGGAGAAAATCTAAAATGAAAAGTGGATAAACAGAACATTTATA
3751   AGTGATCAGTTAATGCCTAAGAGTGAAAGTAGTTCTATTGACATTCCTCAAGATATTTAATATCAACTGCATTAT
3826   GTATTATGTCTGCTTAAATCATTTAAAAACGGCAAAGAATTATATAGACTATGAGGTACCTTGCTGTGTAGGAGG
3901   ATGAAAGGGGAGTTGATAGTCTCATAAAAACTAATTTGGCTTCAAGTTTCATGAATCTGTAACTAGAATTTAATTT
3976   TCACCCCAATAATGTTCTATATAGCCTTTGCTAAAGAGCAACTAATAAATTAAACCTATTCTTTCAAAAAAAAAA
```
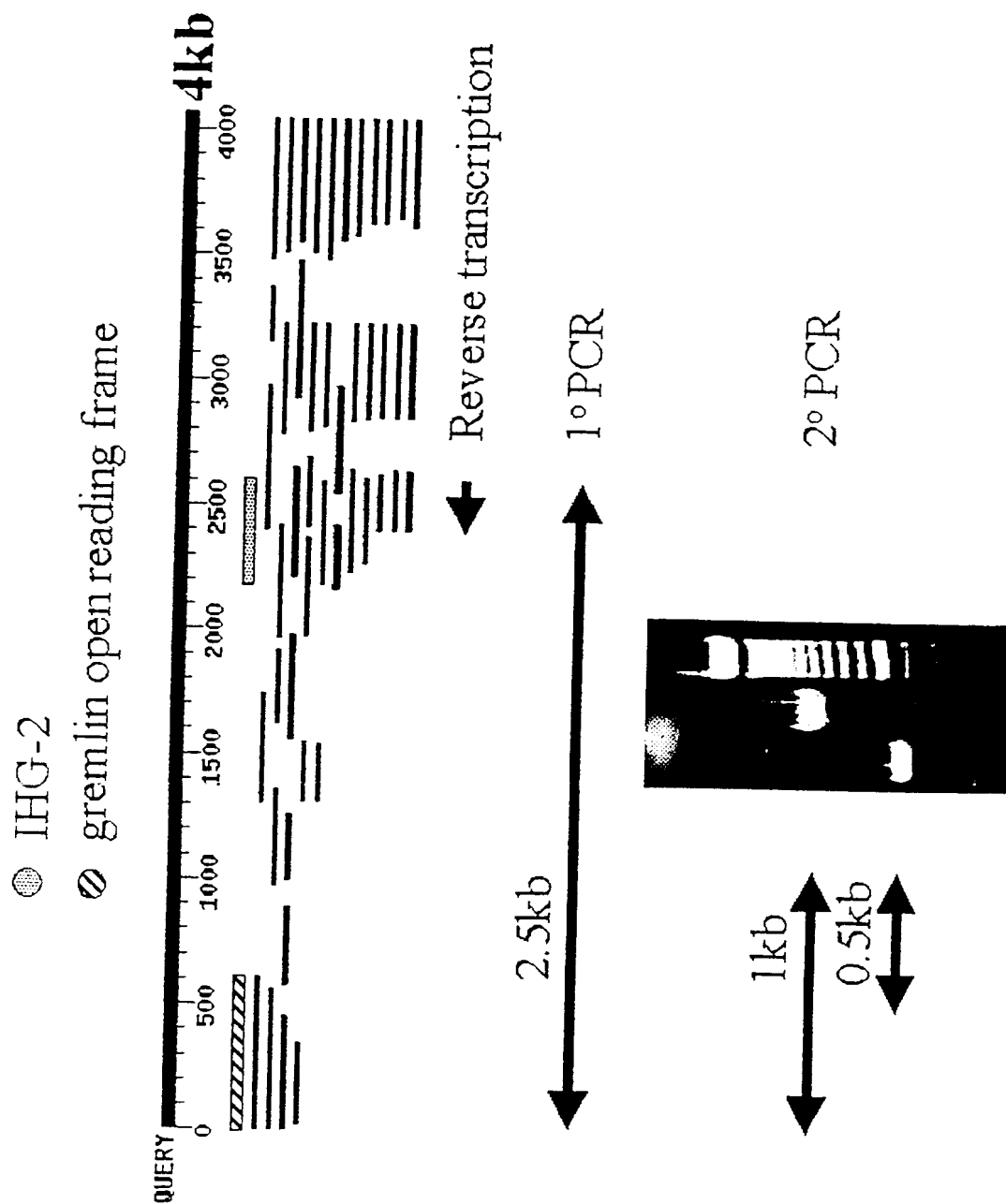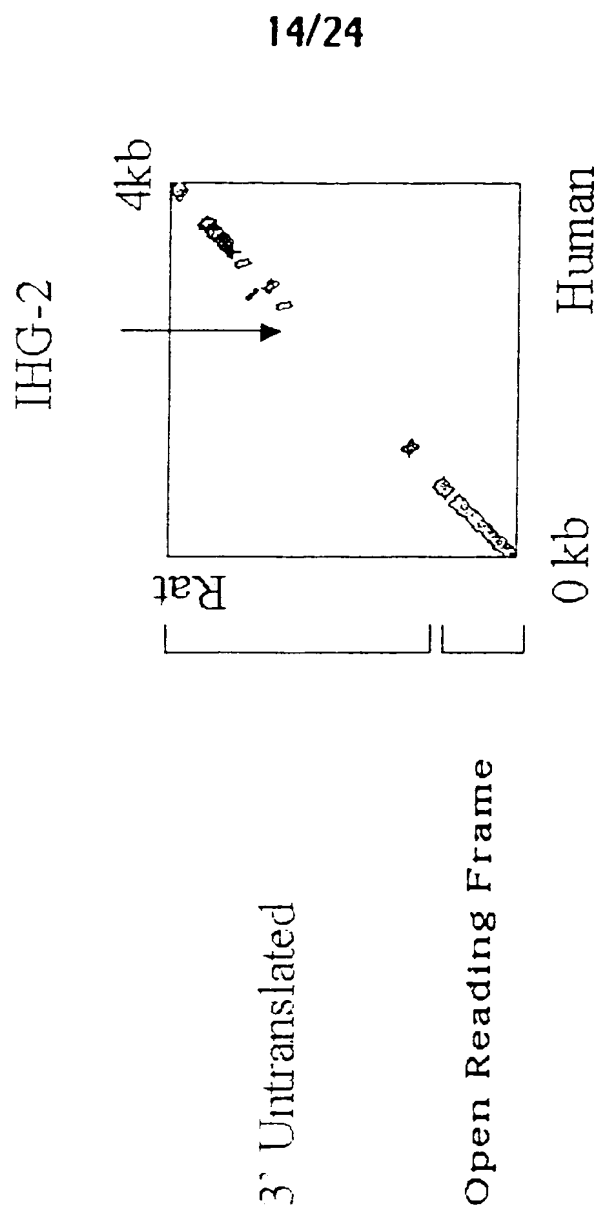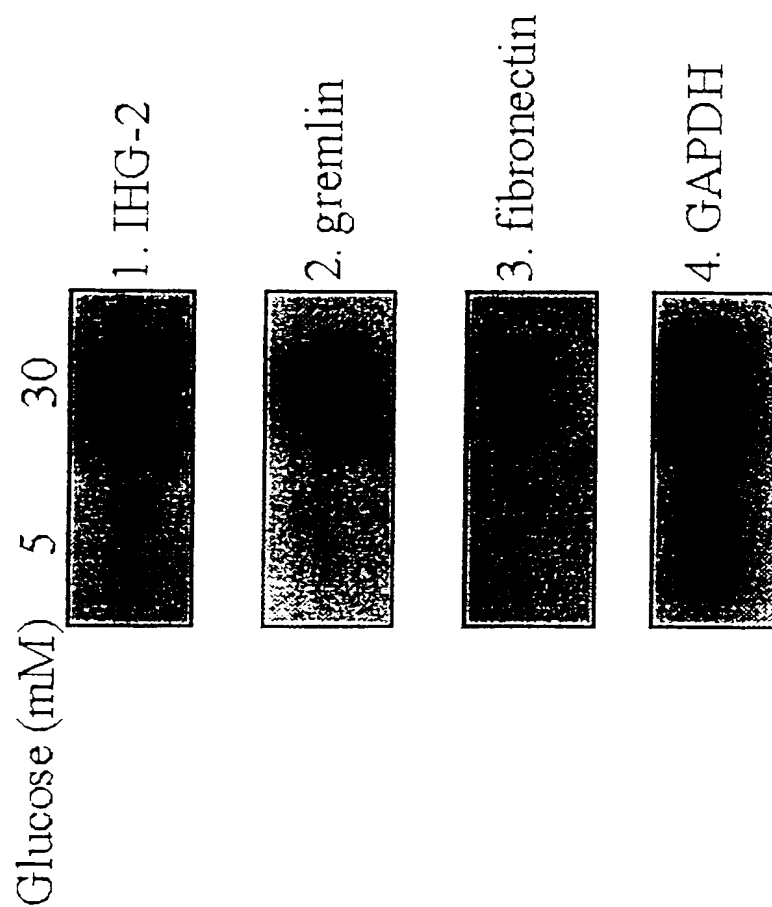
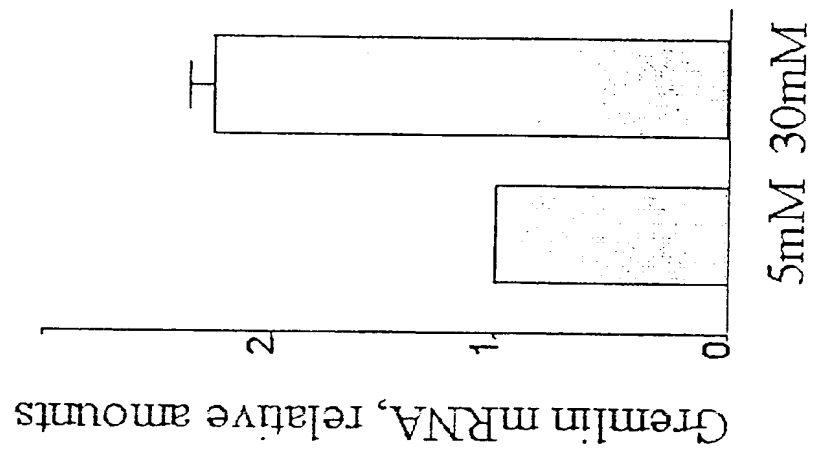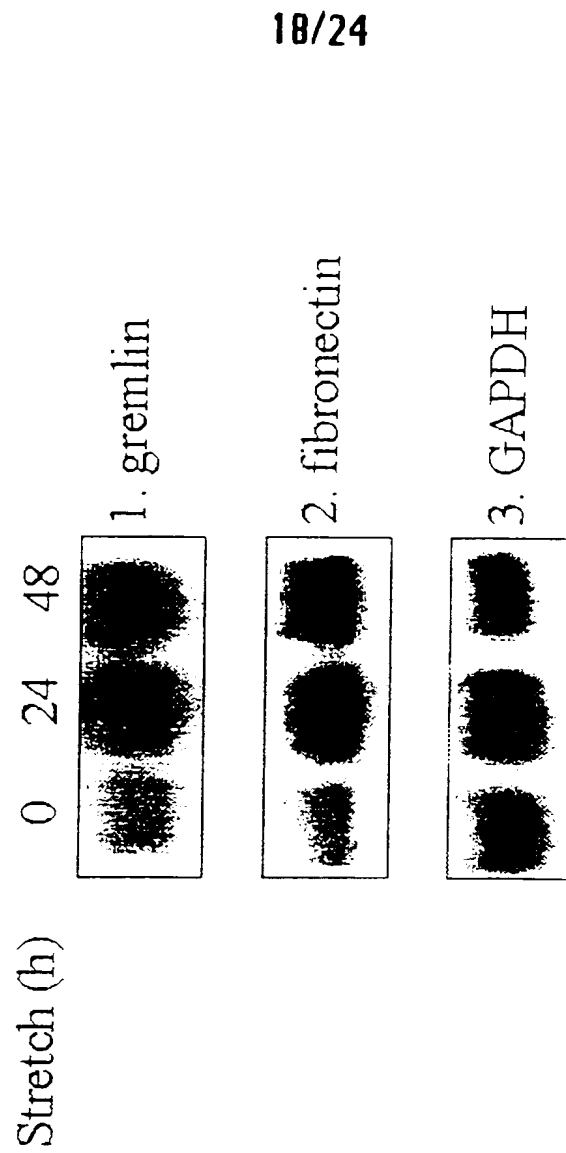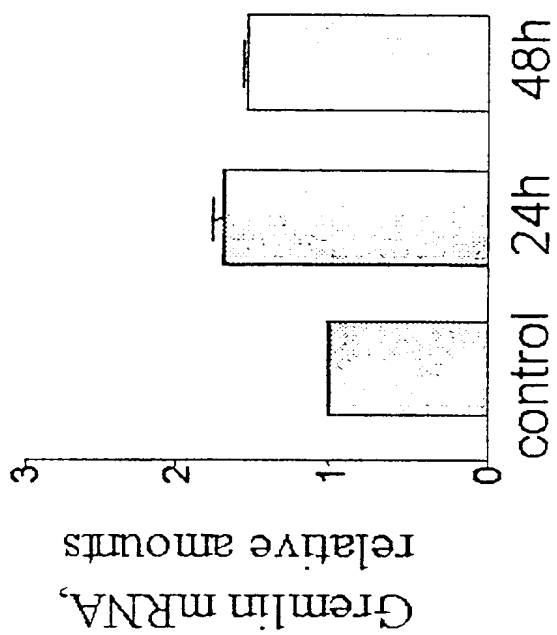(SEQ ID NO: 33)

Fig. 15
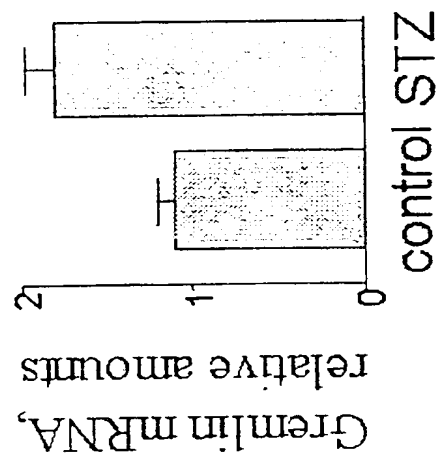
FIG. 16

FIG. 17

FIG. 18

FIG. 19

20/24
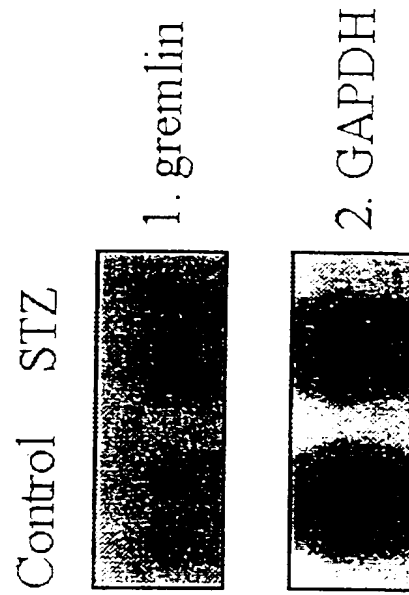
FIG. 20

FIG. 21
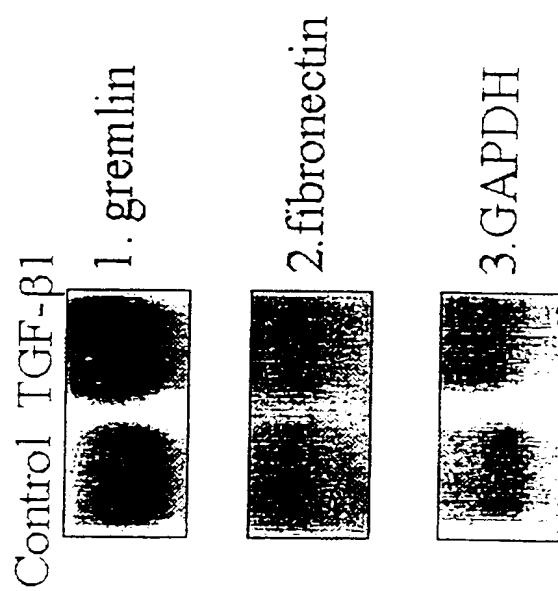
Fig. 22
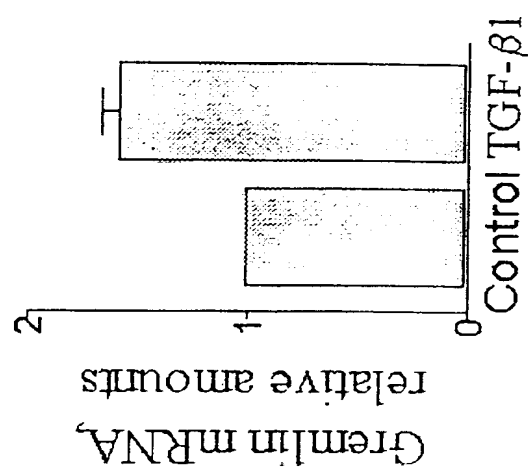
23/24

Fig. 23
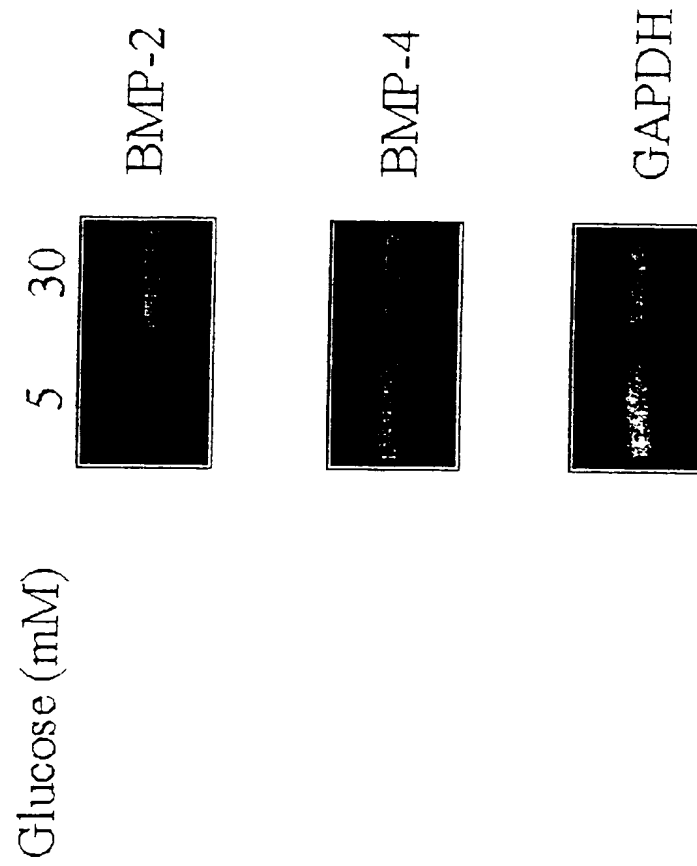
Fig. 24

SEQUENCE LISTING

<110> HiberGen Limited
      University College Dublin, Nat Uni of Ire, Dublin
      Brady, Hugh Redmond
      Godson, Catherine Mary
      Martin, Finian Martin

<120> Identification of genes having a role in the
      presentation of diabetic nephropathy

<130> P00-18-PCT

<140>
<141>

<150> 990157
<151> 1999-02-26

<160> 33

<170> PatentIn Ver. 2.1

<210> 1
<211> 598
<212> DNA
<213> Homo sapiens

<400> 1
ttggaatagt tcttgcttta taaaaatagt actgcgatta aaaaaaaagc acttctgcca 60
aaggaaccat gttccaacac cgcaaacaag gtgttctgct taaacagagt aagatacacc 120
accccatcc atcccttcct tccctgttcc cctcccaact tgagttgtgt cattcgcacc 180
agtgtcctgg gtggtaggga tgctacagcc acctaaggca aggagccctg ggaggtggga 240
gggcttgcat ggttaagcac accagaactg aagcgcaaaa gggtcagctg tcttcatcta 300
gaatctctgg atgttccttc cagaaagcat ccccgatgat atcgcagtgc aagggcactg 360
gctttgtcct ggtccgggtc actgccatct tttttccttc catttctgtt ggcagcttaa 420
tttcttttgt catcacttca tccaccttct gccatatcaa cacagtccct ttcctataca 480
tcggcagctc attattatag ttgatgttga attcagaaaa caaaatctca ttcttgtctg 540
ctgnaagagt tccctgtaat ctcccttggg cttgtactgg tgttagtcca gattgttg 598

<210> 2
<211> 761
<212> DNA
<213> Homo sapiens

<400> 2
ggtcctttaa agtctggttg ctgggrataca ccacgactct tccggtcaaa gcctggggga 60

1

```
tacagaaggg gctrgtcctc aaagtaatcc cgccaataaa acayatagct ggaggcaaac 120
tgggaggyca cgtgagtcat gaactttact ggctcttctt ttaaaccaat tggttttccg 180
cttgwacaca aagctgtact catcactctg tccataacgc gatcacaata tcctctagtt 240
cttccatcac agtctgcgca catttggtca tcagctggag agcacggctg tcattgggtt 300
ttgcaaagtt gtgcttctca gcaaaccgat ggaaattccg gccgtccagc cgnactacca 360
cccagcagtg tgccaggcag gtgtcgtcag cctcgaagtc cctcacgtac tcgaacttgc 420
tttttgccat ggtcgccccc aatctcaggt accgtctcag agtgatggaa atggtggcca 480
aggaatcgtg aaccttaact ttacaggcgc cccacattct acacgcggaa aggaaagggc 540
cagatagccc cgccccggaa gtgttctctt cgtggctact ctagccgtag ggcggtcata 600
gtctctctcg sctctccctg kagttcttaa mcyyccaggg aaaraggatg gaggtttagg 660
ttcctccgtt agcaccttcc acgcttgctt cttcctcctc ccggtctgcg gcaaatcagt 720
rtcacgaggt ttttaaaaat tattttttat ctgctggcct t               761
```

```
<210> 3
<211> 393
<212> DNA
<213> Homo sapiens

<400> 3
atgacacaaa tattaggatt ttatttttac tattatccac cagcaacaag atatcaaaca 60
ctggttctgt gattatttaa tggtgaaaaa gttgaataaa tcaatttagt atacccatat 120
gttggaatat tgagtccatt tttcttttaa aaatcacact ttggaataat tgatgatact 180
ggcaaatgct caagctgagt ggaaaaatat ataaacattg tataggcgaa taattccaat 240
cttgtgcatt ccctgtgtaa acctacatac acaaaaagaa aaagactga aaggaaccat 300
ccacaatgct ttgatcggga aagacggaga aacaaagtgt taattttctt aactatagtt 360
ttnggtgtat tccagatttt ctacaagtta ata                        393
```

```
<210> 4
<211> 435
<212> DNA
<213> Homo sapiens

<400> 4
gtactttgga tttggttaac ctgttttctt caagcctgag gttttatata caaactccct 60
gaatactctt tttgccttgt atcttctcag cctcctagcc aagtcctatg taatatggaa 120
aacaaacact gcagacttga gattcagttg ccgatcaagg ctctggcatt cagagaaccc 180
ttgcaactcg agaagctgtt tttatttcgt ttttgttttg atccagtgct ctcccatcta 240
acaactaaac aggagccatt tcaaggcggg agatatttta aacacccaaa atggttgggt 300
ctgattttca aacttttaaa attcactact gatgattctg cacgctaagg cgaatttggt 360
ccaaacacat aagtgtgtgt gttttgtata cactgtatga ccccacccca aatctttgta 420
ttgtccacat tctcc                                            435
```

```
<210> 5
<211> 273
<212> DNA
```

<210> Homo sapiens

<400> 5
agaagcaatt taggaanccn acagnaaana aatgctgttt tataggagag aaaacacggc 60
acaccaaggt taagtagttt gtagacgatg ttgaataggt tcaggtacag gtcaatgcag 120
tgatgaggaa agcacctang tatacttgac agatagtccc ctttgcttaa cacccaactc 180
ctccaccctg tgcagtttnn cttgtgccag tgatcacagg attcgctgag tgaattacca 240
taattggatt taattcacga aggggatgtt ttc   273

<210> 6
<211> 309
<212> DNA
<213> Homo sapiens

<400> 6
attgatagag gccctgtttc atgacatttc atgagtttca atatgttgtt cagcatgttg 60
tgaggtgact ctcagcccct ttcccactga gatggactgt ggtgatgctg tgagggtgtg 120
actgacacac cttcatgtgc ccaagcatgg gtttgatcac aggtcacatg cagttttttgg 180
catagtaaat gtatcattgt tcttttcctc cctcctaaag gaaacagagg aatccacctg 240
tatgagagtg ccatgtaggg ataaacttaa aggacagatg acacattggt catgttcgtg 300
ataaggaaa   309

<210> 7
<211> 20
<212> DNA
<213> Homo sapiens

<400> 7
accacagtcc atgccatcac   20

<210> 8
<211> 20
<212> DNA
<213> Homo sapiens

<400> 8
tccaccaccc tgttgctgta   20

<210> 9
<211> 20
<212> DNA
<213> Homo sapiens

<400> 9

3

<WO   0050637A1 I >

```
ggtcttcctg gcttaaaggg                                    20


<210> 10
<211> 20
<212> DNA
<213> Homo sapiens

<400> 10
gctggtcagc cctgtagaag                                    20


<210> 11
<211> 20
<212> DNA
<213> Homo sapiens

<400> 11
ccaggagttc caggatttca                                    20


<210> 12
<211> 20
<212> DNA
<213> Homo sapiens

<400> 12
ttttggtccc agaaggacac                                    20


<210> 13
<211> 18
<212> DNA
<213> Homo sapiens

<400> 13
cgaaatcaca gccagtag                                      18


<210> 14
<211> 18
<212> DNA
<213> Homo sapiens

<400> 14
atcacatcca cacggtag                                      18
```

4

<210> 15
<211> 20
<212> DNA
<213> Homo sapiens

<400> 15
ctaagacctg tggaatgggc   20

<210> 16
<211> 21
<212> DNA
<213> Homo sapiens

<400> 16
ctcaaagatg tcattgtccc c   21

<210> 17
<211> 20
<212> DNA
<213> Homo sapiens

<400> 17
atgagccgca cagcctacac   20

<210> 18
<211> 23
<212> DNA
<213> Homo sapiens

<400> 18
ttaatccaaa tcgatggata tgc   23

<210> 19
<211> 21
<212> DNA
<213> Homo sapiens

<400> 19
ctcagcctcc tagccaagtc c   21

<210> 20
<211> 21
<212> DNA

<213> Homo sapiens

<400> 20
gtattgtcca cattctccaa c   21

<210> 21
<211> 20
<212> DNA
<213> Homo sapiens

<400> 21
atgagccgca cagcctacac   20

<210> 22
<211> 21
<212> DNA
<213> Homo sapiens

<400> 22
gtattgtcca cattctccaa c   21

<210> 23
<211> 20
<212> DNA
<213> Homo sapiens

<400> 23
gagagtcaca cgtgtgaagc   20

<210> 24
<211> 19
<212> DNA
<213> Homo sapiens

<400> 24
aggaggatgc aagcacagg   19

<210> 25
<211> 29
<212> DNA
<213> Homo sapiens

<400> 25

6

```
cgcggatcct gcttcttaga cggactgcg                                    29
```

```
<210> 26
<211> 20
<212> DNA
<213> Homo sapiens

<400> 26
tttgctgtac tagcgacacc                                              20
```

```
<210> 27
<211> 18
<212> DNA
<213> Homo sapiens

<400> 27
caagatgaac acagctgg                                                18
```

```
<210> 28
<211> 18
<212> DNA
<213> Homo sapiens

<400> 28
gctcaggata ctcaagac                                                18
```

```
<210> 29
<211> 341
<212> DNA
<213> Rattus sp.

<400> 29
atctccaccc gggttaccaa tgacaatact ttctgcaggc tggagaagca gagtcgtctc 60
tgcatggtca ggccctgtga agctgaccta gaggaaaaca ttaagaaggg caaaaagtgc 120
atccggacgc ctaaaattgc caagcctgtc aagtttgagc tttctggctg caccagtgtg 180
aagacctacc gggctaagtt ctgtgggggtg tgcacggacg gccgctgctg cacaccgcac 240
agaaccacca cactgccggt ggagttcaag tgcccccatg gcgaaatcat gaaaaagaac 300
atgatgttca tcaagacctg tgcctgccat tacaactgtc c                      341
```

```
<210> 30
<211> 341
<212> DNA
<213> Mus sp.
```

```
<400> 30
atctccaccc gagttaccaa tgacaatacc ttctgcagac tggagaagca gagccgcctc 60
tgcatggtca ggccctgcga agctgacctg gaggaaaaca ttaagaaggg caaaaagtgc 120
atccggacac ctaaaatcgc caagcctgtc aagtttgagc tttctggctg caccagtgtg 180
aagacataca gggctaagtt ctgcggggtg tgcacagacg gccgctgctg cacaccgcac 240
agaaccacca ctctgccagt ggagttcaaa tgccccgatg gcgagatcat gaaaaagaat 300
atgatgttca tcaagacctg tgcctgccat tacaactgtc c                      341
```

```
<210> 31
<211> 113
<212> PRT
<213> Rattus sp.
```

```
<400> 31
Ile Ser Thr Arg Val Thr Asn Asp Asn Thr Phe Cys Arg Leu Glu Lys
 1               5                  10                  15


Gln Ser Arg Leu Cys Met Val Arg Pro Cys Glu Ala Asp Leu Glu Glu
            20                  25                  30


Asn Ile Lys Lys Gly Lys Lys Cys Ile Arg Thr Pro Lys Ile Ala Lys
        35                  40                  45


Pro Val Lys Phe Glu Leu Ser Gly Cys Thr Ser Val Lys Thr Tyr Arg
    50                  55                  60


Ala Lys Phe Cys Gly Val Cys Thr Asp Gly Arg Cys Cys Thr Pro His
65                  70                  75                  80


Arg Thr Thr Thr Leu Pro Val Glu Phe Lys Cys Pro His Gly Glu Ile
                85                  90                  95


Met Lys Lys Asn Met Met Phe Ile Lys Thr Cys Ala Cys His Tyr Asn
            100                 105                 110


Cys
```

```
<210> 32
<211> 113
<212> PRT
<213> Mus sp.
```

```
<400> 32
Ile Ser Thr Arg Val Thr Asn Asp Asn Thr Phe Cys Arg Leu Glu Lys
```

```
          1                 5                 10                15

     Gln Ser Arg Leu Cys Met Val Arg Pro Cys Glu Ala Asp Leu Glu Glu
                   20                25                30

     Asn Ile Lys Lys Gly Lys Lys Cys Ile Arg Thr Pro Lys Ile Ala Lys
                   35                40                45

     Pro Val Lys Phe Glu Leu Ser Gly Cys Thr Ser Val Lys Thr Tyr Arg
                50                55                60

     Ala Lys Phe Cys Gly Val Cys Thr Asp Gly Arg Cys Cys Thr Pro His
      65                70                75                80

     Arg Thr Thr Thr Leu Pro Val Glu Phe Lys Cys Pro Asp Gly Glu Ile
                   85                90                95

     Met Lys Lys Asn Met Met Phe Ile Lys Thr Cys Ala Cys His Tyr Asn
                  100               105               110

     Cys
```

```
<210> 33
<211> 4049
<212> DNA
<213> Homo sapiens

<400> 33
gcggccgcac tcagcgccac gcgtcgaaag cgcaggcccc gaggacccgc cgcactgaca 60
gtatgagccg cacagcctac acggtgggag ccctgcttct cctcttgggg accctgctgc 120
cggctgctga agggaaaaag aaagggtccc aaggtgccat cccccgcca gacaaggccc 180
agcacaatga ctcagagcag actcagtcgc cccagcagcc tggctccagg aaccgggggc 240
ggggccaagg gcggggcact gccatgcccg gggaggaggt gctggagtcc agccaagagg 300
ccctgcatgt gacggagcgc aaatacctga agcgagactg gtgcaaaacc cagccgctta 360
agcagaccat ccacgaggaa ggctgcaaca gtcgcaccat catcaaccgc ttctgttacg 420
gccagtgcaa ctctttctac atccccaggc acatccggaa ggaggaaggt cctttcagt 480
cctgctcctt ctgcaagccc aagaaattca ctaccatgat ggtcacactc aactgccctg 540
aactacagcc acctaccaag aagaagagag tcacacgtgt gaagcagtgt cgttgcatat 600
ccatcgattt ggattaagcc aaatccaggt gcacccagca tgtcctagga atgcagcccc 660
aggaagtccc agacctaaaa caaccagatt cttacttggc ttaaacctag aggccagaag 720
aacccccagc tgcctcctgg caggagcctg cttgtgcgta gttcgtgtgc atgagtgtgg 780
atgggtgcct gtgggtgttt ttagacacca gagaaaacac agtctctgct agagagcact 840
ccctattttg taaacatatc tgctttaatg gggatgtacc agaaacccac ctcaccccgg 900
ctcacatcta aaggggcggg gccgtggtct ggttctgact ttgtgttttt gtgccctcct 960
ggggaccaga atctcctttc ggaatgaatg ttcatggaag aggctcctct gagggcaaga 1020
gacctgtttt agtgctgcat tcgacatgga aaagtccttt taacctgtgc ttgcatcctc 1080
```

9

```
ctttcctcct cctcctcaca atccatctct tcttaagttg atagtgacta tgtcagtcta 1140
atctcttgtt tgccaaggtt cctaaattaa ttcacttaac catgatgcaa atgtttttca 1200
ttttgtgaag accctccaga ctctgggaga ggctggtgtg ggcaaggaca agcaggatag 1260
tggagtgaga aagggagggt ggagggtgag gccaaatcag gtccagcaaa agtcagtagg 1320
gacattgcag aagcttgaaa ggccaatacc agaacacagg ctgatgcttc tgagaaagtc 1380
ttttcctagt atttaacaga acccaagtga acagaggaga aatgagattg ccagaaagtg 1440
attaactttg gccgttgcaa tctgctcaaa cctaacacca aactgaaaac ataaatactg 1500
accactccta tgttcggacc caagcaagtt agctaaacca aaccaactcc tctgctttgt 1560
ccctcaggtg aaaagagag gtagtttaga actctctgca taggggtggg aattaatcaa 1620
aaacckcaga ggctgaaatt cctaatacct ttcctttatc gtggttatag tcagctcatt 1680
tccattccac tatttcccat aatgcttctg agagccacta acttgattga taaagatcct 1740
gcctctgctg agtgtacctg acagtaagtc taaagatgar agagtttagg gactactctg 1800
ttttagcaag aratattktg ggggtctttt tgttttaact attgtcagga gattgggcta 1860
ragagaagac gacgagagta aggaaataaa gggrattgcc tctggctaga gagtaagtta 1920
ggtgttaata cctggtagaa atgtaaggga tatgacctcc ctttctttat gtgctcactg 1980
aggatctgag gggaccctgt taggagagca tagcatcatg atgtattagc tgttcatctg 2040
ctactggttg gatggacata actattgtaa ctattcagta tttactggta ggcactgtcc 2100
tctgattaaa cttggcctac tggcaatggc tacttaggat tgatctaagg gccaaagtgc 2160
agggtgggtg aactttattg tactttggat ttggttaacc tgtttttcttc aagcctgagg 2220
ttttatatac aaactccctg aatactcttt ttgccttgta tcttctcagc ctcctagcca 2280
agtcctatgt aatatggaaa acaaacactg cagacttgag attcagttgc cgatcaaggc 2340
tctggcattc agagaaccct tgcaactcga gaagctgttt ttatttcgtt tttgttttga 2400
tccagtgctc tcccatctaa caactaaaca ggagccattt caaggcggga gatattttaa 2460
acacccaaaa tgttgggtct gattttcaaa cttttaaact cactactgat gattctcacg 2520
ctaggcgaat ttgtccaaac acatagtgtg tgtgttttgt atacactgta tgaccccacc 2580
ccaaatcttt gtattgtcca cattctccaa caataaagca cagagtggat ttaattaagc 2640
acacaaatgc taaggcagaa ttttgagggt gggagagaag aaaagggaaa gaagctgaaa 2700
atgtaaaacc acaccaggga ggaaaaatga cattcagaac cagcaaacac tgaatttctc 2760
ttgttgtttt aactctgcca caagaatgca atttcgttaa tggagatgac ttaagttggc 2820
agcagtaatc ttcttttagg agcttgtacc acagtcttgc acataagtgc agatttggct 2880
caagtaaaga gaatttcctc aacactaact tcactgggat aatcagcagc gtaactaccc 2940
taaaagcata tcactagcca aagagggaaa tatctgttct tcttactgtg cctatattaa 3000
gactagtaca aatgtggtgt gtcttccaac tttcattgaa aatgccatat ctataccata 3060
ttttattcga gtcactgatg atgtaatgat atattttttc attattatag tagaatattt 3120
ttatggcaag atatttgtgg tcttgatcat acctattaaa ataatgccaa acaccaaata 3180
tgaattttat gatgtacact ttgtgcttgg cattaaaaga aaaaaacaca catcctggaa 3240
gtctgtaagt tgttttttgt tactgtaggt cttcaaagtt aagagtgtaa gtgaaaaatc 3300
tggaggagag gataatttcc actgtgtgga atgtgaatag ttaaatgaaa agttatggtt 3360
atttaatgta attattactt caaatccttt ggtcactgtg atttcaagca tgttttcttt 3420
ttctccttta tatgactttc tctgagttgg gcaaagaaga agctgacaca ccgtatgttg 3480
ttagagtctt ttatctggtc aggggaaaca aaatcttgac ccagctgaac atgtcttcct 3540
gagtcagtgc ctgaatcttt attttttaaa ttgaatgttc cttaaaggtt aacatttcta 3600
aagcaatatt aagaaagact ttaaatgtta ttttggaaga cttacgatgc atgtatacaa 3660
acgaatagca gataatgatg actagttcac acataaagtc cttttaagga gaaaatctaa 3720
aatgaaaagt ggataaacag aacatttata agtgatcagt taatgcctaa gagtgaaagt 3780
agttctattg acattcctca agatatttaa tatcaactgc attatgtatt atgtctgctt 3840
aaatcattta aaaacggcaa agaattatat agactatgag gtaccttgct gtgtaggagg 3900
atgaaagggg agttgatagt ctcataaaac taatttggct tcaagtttca tgaatctgta 3960
```

10

```
actagaattt aattttcacc ccaataatgt tctatatagc ctttgctaaa gagcaactaa 4020
taaattaaac ctattctttc aaaaaaaaa                                      4049
```

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7   C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7   C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

MEDLINE, STRAND

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HOLMES D I ET AL: "Identification of glucose -regulated genes in human mesangial cells by mRNA differential display." BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, (1997 SEP 8) 238 (1) 179-84. , XP000919240 the whole document	1-7
A	DATABASE EMSTS 'Online! EMBL, Heidelberg; Accesion Number G38490, 24 June 1998 (1998-06-24) MYERS R.M.: "Human STSs 1998" XP002143063 abstract	12

-/--

| X | Further documents are listed in the continuation of box C. | | Patent family members are listed in annex. |

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 July 2000	07/08/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340–2040, Tx. 31 651 epo nl, Fax: (+31-70) 340–3016	Luzzatto, E

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE EMEST 'Online! EMBL,Heidelberg; Accession Number AA071138, **4 October 1996 (1996-10-04)** HILLIER L. ET AL.: "Generation and analysis of 280000 human expressed sequence tags" XP002143064 abstract	12
P,X	MURPHY M ET AL: "Suppression subtractive hybridization identifies high glucose levels as a stimulus for expression of connective tissue growth factor and other genes in human mesangial cells." JOURNAL OF BIOLOGICAL CHEMISTRY, (1999 FEB 26) 274 (9) 5830-4. , XP002142860 the whole document	1-7
P,X	DATABASE EMHUM 'Online! EMBL,Heidelberg; Accesion Number AK000553, 22 February 2000 (2000-02-22) SUGANO S. ET AL.: "NEDO human cDNA sequencing project" XP002143065 abstract	12
P,X	DATABASE GENESEQ 'Online! Derwent; Accession Number Z52443 (WO-A-9957144), 24 February 2000 (2000-02-24) HILLMAN J.L. ET AL.: "New peptides useful for diagnosis,prevention and treatment of cancer and immune disorders" XP002143066 abstract	12
X	DATABASE EMEST 'Online! EMBL, Heidelberg; Accession NUmber N52279, 18 February 1996 (1996-02-18) HILLIER L. ET AL.: "The WashU-Merck EST project" XP002143067 abstract	12

2